The neurobiology of operant learning: biophysical and molecular mechanisms in a hierarchical organization of multiple memory systems

(Die Neurobiologie operanten Lernens: biophysikalische und molekulare Mechanismen in einer hierarchischen Organisation multipler Gedächtnis-Systeme)

Habilitationsschrift

zur Erlangung der Venia Legendi für das Fach Zoologie

Vorgelegt an der Freien Universität Berlin

von

Dr. Björn Brembs

Berlin, 2008

The neurobiology of operant learning: biophysical and molecular mechanisms in a hierarchical organization of multiple memory systems

Content

1. Abstract/Kurzdarstellung

Learning about relationships between stimuli (i.e., classical conditioning) and learning about consequences of one's own behavior (i.e., operant conditioning) constitute the major part of our predictive understanding of the world. This habilitation thesis comprises selected publications on the neurobiological Mechanisms of operant learning and its interactions with classical learning. A prerequisite for operant learning is spontaneous behavioral variability for which we found first neurobiological determinants. We discovered modifications in the biophysical membrane properties of identified *Aplysia* neurons in which operant behavior and reward converge. The processes modifying *Drosophila* neurons during pure operant learning were genetically different from those during classical learning. As soon as predictive stimuli are present in operant learning situations, these stimuli become equivalent to classical stimuli not only with respect to their independence from the behavior with which they were learned, but these composite experiments cannot be distinguished genetically from classical experiments any more. Operant control over such predictive, classical stimuli facilitates learning of these stimuli. At the same time, such operantly facilitated of classical learning inhibits operant learning. The putative function of this inhibition is to prevent premature habit formation from interfering with the generalization of classical memories.

Den Grossteil unseres prädiktiven Verständnisses der Welt gewinnen wir aus dem Lernen von Zusammenhängen in der Umwelt (klassische Konditionierung), sowie dem Lernen aus Verhaltenskonsequenzen (operante Konditionierung). Diese Habilitationsschrift umfasst meine Arbeiten zu den neurobiologischen Mechanismen operanten Lernens und dessen Interaktion mit klassischem Lernen. Grundvoraussetzung für operantes Lernen ist Spontanverhalten. Unsere Untersuchungen ergaben erste Hinweise auf die Art und Weise wie Gehirne Spontanverhalten generieren. *Aplysia* Neurone in denen operantes Verhalten und Belohnung konvergieren zeigten durch operantes Lernen hervorgerufene biophysikalische Veränderungen. In *Drosophila* zeigten wir, dass die Vorgänge die Fliegen-Neurone beim rein operanten Lernen verändern, auf anderen genetischen Mechanismen beruhen, als die Vorgänge beim klassischen Lernen. Sobald jedoch prädiktive Reize in operanten Lernsituationen vorkommen, werden diese Reize klassischen Reizen nicht nur im Hinblick auf deren Verhaltensunabhängigkeit äquivalent, sondern diese Experimente sind auch genetisch nicht mehr von klassischen Lernexperimenten zu unterscheiden. Operante Kontrolle über diese prädiktiven, klassischen Reize fördert das Lernen über diese Reize. Gleichzeitig inhibiert dieses operant geförderte klassische Lernen das operante Lernen. Funktion dieser Inhibition ist es zu verhindern, dass operantes Gedächtnis (Gewohnheiten) mit der Generalisierung von klassischem Gedächtnis interferiert.

---

2. Summary of the submitted publications

This tome is meant to collect my research over the last six years and hand it in as the cumulative "Habilitationsleistung" at the Freie Universität Berlin. For this purpose I will now briefly list the selected publications and explain their connection to each other as well as to my current research. The neurobiology of spontaneous behavior and the operant learning it allows have been my research topic from the very beginning. With neurobiology ideally being studied at the genetic, physiological and behavioral level, two complementing model systems were chosen which both exhibited spontaneous behavior and operant learning, and where one was more accessible genetically and the other was more accessible physiologically.

Because of the superior physiological access to the individual neurons which generate behavior in the marine snail *Aplysia*, we extended previous work in this model system to also incorporate *in vivo* operant learning (**Brembs et al., 2002**) and an *in vitro* preparation in which both operant and classical processes can be studied simultaneously (**Brembs et al., 2004**). These experiments on *Aplysia* feeding behavior revealed how an identified neuron (B51) which is involved in determining what behavior is generated is modified by dopamine-mediated contingent reward such that future behavior will be biased towards the rewarded behavior. In a single-cell analogue of operant learning, we demonstrated how activity-dependent plasticity changed input resistance and burst threshold in B51 only in neurons which had received iontophoretic pulses of dopamine contingent with bursting activity and not in unpaired neurons (**Brembs et al., 2002**). Because B51 is active only late during the behavior, it cannot be critically involved in the generation of the behavior, only in determining what behavior is to be produced. Therefore, part of my research effort is currently focused on the optophysiology of spontaneously active isolated *Aplysia* buccal ganglia to investigate the circuitry involved.

Because of the superior genetic accessibility of the fruit fly *Drosophila*, we used transgenic and wildtype flies to study the neurobiology of spontaneous behavior and operant learning in both freely behaving and tethered *Drosophila*. Stricken by the spontaneous outbursts of aggression and the subsequent development of strict territoriality in freely behaving flies, we initiated the research on the neurobiological determinants of aggressive behavior (**Baier et al., 2002**). Interestingly, two of these determinants were the biogenic amines octopamine and dopamine, which later turned out to be involved in processing appetitive and aversive stimuli, respectively, during learning. Receptors for both amines are preferentially expressed in the mushroom-bodies and blocking output from this neuropil reduces the level of aggression. Another important factor was β-alanine, the concentration of which is regulated by the actions of the *black* and *ebony* genes, respectively. Further characterizing the *black* gene locus, we found that $black^{1}$ mutant flies lack a pyridoxal-5-phosphate, PLPdependent decarboxylase, *Dgad2*. This mutant, besides showing reduced levels of aggression, also behaves abnormally in Buridan's paradigm, which cannot be explained by a lack of first order visual function as no electroretinogram or target recognition defects were detected (**Phillips et al., 2005**). The *Dgad2* gene is an excellent example for the pleiotropy of genes involved in behavior which warrants more sophisticated inter-

ventions than constitutive gene knock-outs. Further demonstrating this fact is a study which involved stationary flying *Drosophila* (**Brembs et al., 2007**). Combining lines of evidence from several topics, this study investigated the influence of octopamine and its precursor, tyramine, on flight performance. With octopamine being critically involved in flight performance in several insect species as well as in initiating aggressive behaviors and in mediating appetitive stimuli during learning, it became necessary to find out if mutants lacking octopamine (*Tyramine β-Hydroxylase* mutants) are suited for learning experiments in tethered flight at the flight simulator. Our transgenic and pharmacological treatments revealed a complex, degenerate orchestration of flight performance in which lack of either octopamine or tyramine could be compensated for and only an ablation of all tyraminergic/octopaminergic neurons completely abolished sustained flight. These results are best explained with a wide range of subpopulations of tyraminergic and octopaminergic neurons which each contribute to any of the observed phenotypes in aggression, motor control and learning.

Wildtype flies, tethered in stationary flight as in the previous experiments, can fly continuously for several hours. Attached to a torque meter, they reveal a striking variability in their turning behavior. Analyzing the temporal structure of the yaw torque of wildtype flies in various situations with and without re-afferent feedback revealed that the variability in the behavior of the flies is best explained by a non-linear mechanism (**Maye et al., 2007**). This result rules out simple stochastic processes and instead suggests that even seemingly random variability in the fly's behavior is generated spontaneously and endogenously by the fly's brain. These data dovetail nicely with a number of neurobiological, evolutionary and ecological findings which indicate that spontaneous behavioral variability is an evolved trait with a neurobiological basis (**Brembs, 2008, subm**.). Because spontaneous behavior is also a prerequisite for operant learning, we studied various forms of operant learning with tethered *Drosophila* at the torque meter.

To study learning in tethered *Drosophila*, a rigorous breeding regime is required, as well as sophisticated mechanical setup which allows the exquisite control of the fly's environment. These experimental procedures have recently been described for the first time in a peer-reviewed video publication (**Brembs, 2008**). This setup allowed us to observe a peculiar effect in higher-order learning which had already been observed in simple pattern learning before: operant control of external stimuli facilitates learning about these stimuli (**Brembs and Wiener, 2006**). In this case, operant control of the colors which determined which one of two visual patterns was being punished, allowed the animals to solve this occasion setting situation, whereas classical presentation of the colors did not lead to significant learning. The mushroom-bodies were not required for the operant facilitation of occasion setting and just as wildtype flies, flies with blocked mushroom-body output also failed the classical version. Occasion setting leads to a form of context-dependent memory: in one occasion (e.g. green coloration), one of two patterns is punished, in the other occasion (e.g., blue coloration), the other pattern is punished. Flies which have learned this relationship have developed a pattern-memory which is dependent on the color context. Further exploiting this new occasion setting paradigm as well as a previously developed paradigm to study context-independent memory (i.e., context generalization), we found that generaliza-

tion and discrimination rely on two different parameters of the colors used (**Brembs and Hempel de Ibarra, 2006**). Specifically, generalization occurs only if the chromaticity is sufficiently similar, whereas discrimination learning relies on brightness differences.

Generalization and discrimination are also at the heart of the set of experiments which aimed at understanding the genetic basis for operant learning and how operant learning interacts with other forms of learning, such as classical learning. Our genetic study showed a double dissociation of the molecular processes involved in operant and classical learning (**Brembs and Plendl, 2008, re-subm**.). Specifically, the *rutabaga* (*rut*-)adenylyl cyclase was required for classical learning, but not for operant learning, whereas protein kinase C (PKC) was required for operant but not for classical learning. Importantly, this double dissociation could only be observed if the operant learning paradigm did not include any predictive stimuli at all ('pure' operant learning). As soon as a predictive stimulus was present, learning about this stimulus dominated the experiment. This result corroborated and extended a previous experiment from my diploma and PhD thesis where wildtype animals generalized such an operantly controlled stimulus across behavioral contexts. In other words, predictive stimuli contained in operant learning situations become equivalent to classical stimuli not only because they are acquired independently of the behavior with which they were controlled during training, but also because of the genes required for the learning task. Because the mushroom-bodies are involved in some forms of generalization, I trained flies with blocked mushroom-body output in a situation with both operant and classical predictors and then tested them for any operant component and generalization of any classical component (**Brembs, 2008, in prep**.). The results indicate that the dominance of the classical stimuli in such composite learning situations is mediated by the mushroom-bodies inhibiting operant learning. Corroborating the results from higher-order learning, the mushroom-bodies seem not to be involved in the facilitation of classical learning in these experiments either. Thus, these data are consistent with the hypothesis that there are reciprocal interactions between a *rut*-dependent classical system and a PKC-dependent operant system. The classical system dominates in learning situations where predictive stimuli are present and inhibits operant learning via the mushroom-bodies. A component of the operant system (operant behavior) facilitates the classical system via unknown, non-mushroom-body pathways. The proposed function of this reciprocal arrangement is to prevent the operant system from interfering with generalization of classical memory. In this view, the interfering action of the operant system consists of storing behavioral memories as habits.

3. Zusammenfassung der eingereichten Arbeiten

Die hier vorgelegten Publikationen stehen exemplarisch für meine experimentellen und konzeptionellen Forschungen der letzten sechs Jahre und werden als kumulative Habilitationsleistung an der Freien Universität Berlin eingereicht. Die zusammengestellten Arbeiten sollen hier kurz vorgestellt werden um ihren Zusammenhang und ihre Stellung innerhalb meines wissenschaftlichen Konzeptes zu erläutern. Kernpunkt dieses Konzepts war schon von Beginn an die Neurobiologie von Spontanverhalten und operantem Lernen. Da Neurobiologie mindestens gleichzeitig auf der genetischen, physiologischen und Verhaltens-Ebene studiert werden sollte, wurden zwei komplementäre Modellsysteme ausgewählt, die beide Spontanverhalten und operantes Lernen zeigen. Eines ist jedoch eher ein genetisches Modellsystem, das andere eher ein physiologisches.

Aufgrund des einfacheren physiologischen Zugangs zu den einzelnen Neuronen die das Verhalten der marinen Nacktschnecke *Aplysia* generieren, erweiterten wir dieses System um ein operantes Lernexperiment am intakten Tier (**Brembs et al., 2002**) und um eine *in vitro* Präparation in der operante und klassische Vorgänge gleichzeitig untersucht werden können (**Brembs et al., 2004**). Diese Experimente am Fressverhalten von *Aplysia* zeigten wie ein einzelnes Neuron (B51; Aktivität in B51 trägt wesentlich zu der Entscheidung bei, welches Verhalten generiert wird) durch Dopamin-vermittelte Belohnung so modifiziert wird, dass das belohnte Verhalten häufiger auftritt. Experimente an einem Einzellzell-Analog operanten Lernens demonstrierten wie Aktivitätsabhängige Plastizität den Eingangswiderstand und die Aktivitäts-Schwelle von B51 verändert. Diese Modifikationen traten nur dann auf, wenn iontophoretische Dopamingaben direkt auf Aktivität in B51 folgten und nicht, wenn sie nicht mit Aktivität in B51 gekoppelt waren (**Brembs et al., 2002**). Weil B51 erst spät während des Verhaltens aktiv ist, kann es nicht an der Initiierung des Verhaltens beteiligt sein, sondern nur daran, welches verhalten produziert wird. Daher besteht ein Teil eines laufenden, DFG-geförderten Projektes darin, die Aktivität aller Neurone im spontan Verhalten generierenden, isolierten Buccalganglion optophysiologisch abzuleiten und die zugrundeliegenden Netzwerke zu untersuchen.

Aufgrund der besseren genetischen Möglichkeiten in der Taufliege *Drosophila* wurden wildtypische und transgene Fliegen herangezogen um die Neurobiologie des Spontanverhaltens und des operanten Lernens sowohl in frei fliegenden/laufenden Tieren als auch in fixierten Tieren erforscht. Angespornt durch die spontanen Ausbrüche von Aggressivität und der darauffolgenden Entwicklung von Territorialität in sich frei bewegenden Fliegen starteten wir die neue Forschungsrichtung der neurobiologischen Faktoren aggressiven Verhaltens (**Baier et al., 2002**). Interessanterweise waren zwei dieser Faktoren die biogenen Amine Oktopamin und Dopamin, von denen man später herausfinden sollte dass sie bei der Vermittlung von appetitiven und aversiven Reizen beim Lernen eine entscheidende Rolle spielen. Rezeptoren für beide Amine sind präferentiell in den Pilzkörpern exprimiert. Eine genetische Blockade des Ausgangs dieses Neuropils reduziert aggressive Verhaltensweisen in der Fliege. Als weiterer wichtiger Faktor erwies sich β-Alanin, dessen Konzentration über die Aktivität der Gene *black* und *ebony* reguliert wird. Daher begannen wir den

black Genlocus weiter zu charakterisieren. Wir fanden, dass Fliegen mit der *black*^{1} Mutation keine nachweisbare Aktivität der Pyroxidal-5-Phosphat, PLP-abhängige Decarboxylase, *Dgad2*, zeigen. Diese Mutanten zeigen neben reduziertem Aggressionsverhalten auch Veränderungen des Verhaltens im Buridan-Paradigma, die nicht durch einen Verlust primärer Sehfunktion erklärt werden können, da sie keine Defekte im Elektroretinogramm oder in der Zielerkennung aufweisen (**Phillips et al., 2005**). Das *Dgad2* Gen ist ein exzellentes Beispiel für Pleiotropie, typisch für so viele Verhaltens-relevante Gene und der Grund, warum genetische Verhaltensstudien oft raffiniertere genetische Manipulationen erfordern als konstitutive Gen-Ausschaltung. Eine weitere unserer Studien demonstriert diese Problematik auch (**Brembs et al., 2007**). Diese Arbeit untersuchte den Einfluss von Oktopamin und dessen Vorstufe Tyramin auf die Flugleistung der Tiere. Da Oktopamin in mehreren Insekten Arten eine prominente Rolle bei der Kontrolle des Flugverhaltens spielt, wichtig für normales Aggressionsverhalten ist und darüber hinaus auch essentiell beim Lernen mit appetitiven Reizen involviert ist, drängte es sich auf, die bereits bestehenden Null-Mutanten für das *Tyramin-β-Hydroxylase* Gen auf deren Leistung im stationären Flug zu untersuchen, da in diesen Tieren kein Oktopamin mehr nachweisbar ist. Unsere transgenen und pharmakologischen Manipulationen deckten eine komplexe, degenerierte Orchestrierung der Flugleistung auf, in der sowohl das Fehlen von Oktopamin als auch das von Tyramin alleine kompensiert wurde, und nur eine Ablation aller tyraminergen und oktopaminergen Neurone zum vollständigen Verlust ausdauernden Fluges führte. Diese Ergebnisse lassen sich erklären, wenn man annimmt, dass die oktopaminergen und tyraminergen Systeme aus mehreren Subpopulationen von Neuronen bestehen, die überlappend zu den beobachteten Phänotypen in Aggression, Motorkontrolle und Lernen beitragen.

Fixiert man wildtypische Fliegen wie in den letzten Experimenten, so können sie stundenlang fliegen. Mit einem Drehmomentmessgerät kann man beobachten, dass diese Tiere unter anderem ständig hochvariables Steuerverhalten um ihre Hochachse zeigen (Gierungs-Drehmoment). Wir haben die zeitliche Struktur dieses Drehmoment-Signals sowohl in völlig gleichförmiger Reizsituation, als auch mit verschiedenen, operant kontrollierten visuellen Reizen untersucht (**Maye et al., 2007**). Es stellte sich heraus, dass diese Variabilität Anzeichen für einen nicht-linearen Mechanismus enthält. Dieses Ergebnis schliesst einfachen Zufall als Ursache für die Variabilität im Verhalten aus und legt stattdessen nahe, dass selbst scheinbar zufällige Verhaltensentscheidungen vom Fliegengehirn endogen und spontan gefällt werden. Dieser Befund reiht sich nahtlos in eine ganze Reihe von neurobiologischen, evolutionstheoretischen und ökologischen Arbeiten ein, die Spontanverhalten als evoluierte Eigenschaft mit neurobiologische Grundlage sehen (**Brembs, 2008, subm**.). Spontanverhalten ist auch eine der Grundlagen von operantem Lernen und so untersuchten wir eine ganze Reihe operanter Lernphänomene an stationär fliegenden Fliegen am Drehmoment Kompensator.

Damit fixierte *Drosophila* Fliegen konsistent lernen, müssen sie nach einem strengen Zuchtprogramm aufgezogen werden. Um die Reizumgebung des Tieres vollständig unter die Kontrolle des Experimentators zu bringen, bedarf es eines ausgeklügelten mechanischen Aufbaus mit speziell angefertigter Elektronik und Software. Das Zuchtprotokoll, sowie der Versuchsaufbau wurden kürzlich zum ersten mal ausführlich in einer begutachteten Video-

Publikation detailliert beschrieben (**Brembs, 2008**). Die dort gezeigte Anordnung erlaubte es uns einen auffälligen Effekt auch bei einem Lernenvorgang höherer Ordnung zu beobachten, der bereits hinlänglich aus einfachen Lernexperimenten bekannt war: die operante Kontrolle über externe Reize fördert das Lernen über diese Reize (**Brembs and Wiener, 2006**). In diesem Fall ermöglichte die operante Kontrolle über Farbreize, die bestimmten welches von zwei visuellen Mustern bestraft wird, diese ‚*Occasion Setting*' Situation erfolgreich zu lösen. Klassische Präsentation der Farbreize führte nicht zu einem Lernerfolg. Die Pilzkörper waren für diese operante Förderung von *Occasion Setting* nicht wichtig und genau wie wildtypische Tiere konnten Tiere mit blockiertem Pilzkörper-Ausgang auch das klassische *Occasion Setting* nicht lernen. *Occasion Setting* führt zu einer Art von Kontext-abhängigem Gedächtnis: bei der einen Gelegenheit (z.B. grün) wird eines von zwei visuellen Mustern bestraft, bei der anderen (z.B. blau) wird das andere bestraft. Fliegen die dieses Verhältnis lernen, haben ein Mustergedächtnis entwickelt, das vom Farbkontext abhängt. Mit diesem neuen *Occasion Setting* Experiment sowie mit einem bereits bestehenden Experiment das zu Kontext-unabhängigem Gedächtnis führt (d.h. Kontext-Generalisierung), konnten wir herausfinden, dass Generalisierung und Diskriminierung bei Fliegen von zwei unterschiedlichen Parametern der Farben abhängt (**Brembs and Hempel de Ibarra, 2006**). Generalisierung trat nur auf, wenn die Chromatizität der Farben ähnlich genug ist, während Diskriminierung auf Helligkeitsunterschieden zwischen den beiden Farben beruhte.

Die Prozesse Generalisierung und Diskriminierung sind auch zentral für eine Versuchsreihe, die darauf abzielt die genetischen Grundlagen des operanten Lernens und dessen Interaktionen mit anderen Lernformen wie dem klassischen Lernen zu verstehen. Unsere genetische Studie fand eine doppelte Dissoziation der molekularen Mechanismen des operanten und des klassischen Lernens (**Brembs and Plendl, 2008, re-subm**.). Die *rutabaga* (*rut*-)Adenylat-Zyklase wird für das klassische jedoch nicht für das operante Lernen benötigt. Umgekehrt ist die Protein Kinase C (PKC) für das operante jedoch nicht das klassische Lernen essentiell. Ein wichtiger Befund hierbei war zudem, dass diese doppelte Dissoziation nur dann galt, wenn das operante Experiment frei von prädiktiven äusseren Reizen war (‚rein' operantes Lernen). Sobald ein prädiktiver Reiz eingebunden wurde, dominierte das Lernen über diesen Reiz das Experiment. Dieses Ergebnis bestätigte und erweiterte Resultate aus meiner Diplom- und Doktorarbeit, wo ich herausgefunden hatte, dass Fliegen einen so operant gelernten Reiz über Verhaltenskontexte hinweg generalisieren können. Mit anderen Worten, prädiktive Reize in operanten Lernsituationen werden klassischen Reizen nicht nur im Hinblick auf deren Verhaltensunabhängigkeit äquivalent, sondern diese Experimente sind auch genetisch nicht mehr von klassischen Lernexperimenten zu unterscheiden. Da bereits bekannt war, dass die Pilzkörper eine Rolle bei der Kontext-Generalisierung spielen, wurden Tiere mit blockiertem Pilzkörper in einer solchen Situation (operantes Lernen mit prädiktivem Reiz) trainiert und nach dem Training auf die operante Komponente sowie die Generalisierung der klassischen Komponente getestet (**Brembs, 2008, in prep**.). Die Ergebnisse legen nahe, dass die Dominanz von klassischen Reizen in operanten Lernsituationen von den Pilzkörpern vermittelt wird. Dass die Pilzkörper auch in diesen Experimenten nicht an der operanten Förderung klassischen Lernens beteiligt sind, bestätigt die Ergeb-

nisse der *Occasion Setting* Experimente. Diese Daten passen zu der Hypothese, dass es reziproke, hierarchische Interaktionen zwischen einem *rut*-abhängigen, klassischen System und einem PKC-abhängigen, operanten System gibt. Das klassische System dominiert Lernsitationen mit sowohl operanten als auch klassischen Anteilen und inhibiert operantes Lernen mittels der Pilzkörper. Eine Komponente des operanten Systems, operantes Verhalten, fördert die Funktion des klassischen Systems über unbekannte, nicht-Pilzkörper Bahnen. Die putative Funktion dieser reziproken Organisation ist es, eine Behinderung der Generalisierung von klassischem Gedächtnis durch das operante System zu verhindern. In dieser Sichtweise besteht die mögliche Behinderung der Generalisierung aus dem Abspeichern von operanten Verhaltens-Gedächtnissen als Gewohnheiten.

4. Introduction

Few questions are more fundamental than that of how the brain works. Few puzzles are more complex to solve than that of how the brain works. Therefore, it was a clever decision in the last half of the 20th century to turn to less complex model systems to come closer to a solution. After all, worms, snails or insects have far fewer neurons than mammals and most even have a number of other technical advantages as well, yet still possess brains that are capable of solving all the basic problems of life: finding food, mates, procreating, surviving. Producing such adaptive behavior (i.e., increasing fitness) is the main function of brains. Rephrasing Dobzhansky, one may say that nothing in the neurosciences makes sense except in the light of behavior. Smelling, hearing, or seeing would remain senseless if there were no behavior to make use of the perceptions. Behavior is the key to understanding how the brain works. Using the ingenious approach of limiting the behavioral options of the animal, invertebrate behavioral neuroscience unraveled large parts of how animals perceive external stimuli and how they react to them. In fact, our progress in this enterprise has been so overwhelming that until recently some researchers still expressed the view that reacting to external stimuli is all a brain needs to do: "brain function is ultimately best understood in terms of input/output transformations and how they are produced" (Mauk, 2000). So pervasive was this view that any behavior was commonly referred to as a 'response', implicitly assuming a triggering stimulus.

4.1. Spontaneous behavioral variability

However, freely moving animals show highly variable behavior and many experimental preparations are so successful precisely because they limit this variability. Is this variability just noise or is it under the control of the animal? If spontaneous behavioral variability is under the control of the animal, what are its ultimate and proximate causes? What is the evolutionary benefit of behavioral variability and how does the brain generate variable behavior? There is now accumulating evidence from various biological disciplines that spontaneous behavioral variation is an adaptive trait, the mechanistic basis of which can be studied in any suitable model system. For instance, several evolutionary and ecological studies have found behavioral variability to confer a range of fitness benefits or contribute to trophic network stability (e.g., Driver and Humphries, 1988; Grobstein, 1994; Belanger and Willis, 1996; Brembs, 1996a; Miller, 1997; Jablonski and Strausfeld, 2001; Glimcher, 2003; McNamara et al., 2004; Neuringer, 2004; Platt, 2004; Glimcher, 2005; Shultz and Dunbar, 2006; Okuyama, 2007). The fitness benefits easily exemplified in pursuit/evasion contests where any predictable strategy will be exploited (Grobstein, 1994; Brembs, 1996a; Jablonski and Strausfeld, 2000, 2001; Glimcher, 2005). Spontaneous behavioral variability is not due to random noise in the brain but is actively generated by the brain (Maye et al., 2007). Interestingly, much of the behavioral variability is generated not during, but before the behavior is actually performed (Churchland et al., 2006). Finally, at least in humans, much of the behavioral variability can be attributed to fluctuations in the so-called "default network" (Fox et al., 2007). Thus, behavioral variability is an evolved trait, actively generated by the brain with a genetic

basis, much as any of the sensory or sensorimotor processes currently under intense investigation in the neurosciences.

Among the many evolutionary benefits of spontaneous behavioral variability is its capability to confer a sense of agency to the animal via the re-afference principle (von Holst and Mittelstaedt, 1950; Heisenberg, 1983, 1994). Behavioral output (efference) is compared with incoming sensory input (afference) to detect when the animal is the one authoring environmental change. The knowledge derived from such computations is then used to control sensory input (Wolf and Heisenberg, 1991; Wegner, 2002; Todorov, 2004; Webb, 2004; Bays et al., 2006). Experimental studies commonly use operant learning to study this constantly ongoing tripartite operant process of spontaneous behavior, re-afferent feedback and agency.

4.2. Operant behavior and learning

The first experiments into the mechanistical basis of operant behavior and learning was initiated already early in the 20th century by psychologists like Thorndike (1911), Watson (1928) and Skinner (1938). It was first distinguished from Pavlovian or classical learning as “two forms of conditioned reflexes” 80 years ago (Miller and Konorski, 1928). Ever since then, a recurrent concern has been the issue of whether one biological process can account for both of them (Skinner, 1935; Konorski and Miller, 1937b, a; Skinner, 1937; Rescorla and Solomon, 1967; Gormezano and Tait, 1976; Heisenberg et al., 2001; Brembs et al., 2002; Dayan et al., 2006; Lorenzetti et al., 2006b). The discussion has varied between early singular concepts (Guthrie, 1952; Hebb, 1956; Kimmel, 1965; Prokasy, 1965; Miller and Konorski, 1969), later multi-process views (Rescorla and Solomon, 1967; Trapold and Overmier, 1972; Gormezano and Tait, 1976; Rescorla, 1987; Corbit and Balleine, 2005; Blaisdell et al., 2006; Park et al., 2006) and a variety of unified theories (Friston et al., 1994; Donahoe et al., 1997).

In the neurosciences, with the success of research into the mechanisms of classical conditioning, the focus has understandably shifted away from operant learning. It is an understandable shift, because nearly every learning situation seems to involve a dominant classical component (Rescorla, 1987; Brembs and Plendl, 2008, re-subm.) and classical conditioning offers the unique advantage to quickly and easily get at the biological processes underlying learning and memory: the animals are usually restrained, leaving only few degrees of freedom and the stimuli can be traced to the points of convergence in the brain where the learning must be taking place. Today, it is being recognized that, at an adaptive level, cognitive capacities such as those involved in encoding the predictive relations between stimuli, can be of little *functional* value to a hypothetical, purely Pavlovian organism. For instance, one can imagine any number of situations which require the animal to modify, even to withhold or reverse, the direction of some behavior in order to solve the situation. Such situations demand greater behavioral flexibility than the system mediating classical conditioning provides. Moreover, using the re-afference principle, operant behavior underlies the distinction between observing and doing, i.e. differentiating between self and non-self. One almost iconographic example of such behavior is to perform various spontaneous movements in front of a mirror to detect whether it is us we are perceiving. Even animals

perform these movements (Reiss and Marino, 2001; Plotnik et al., 2006). This automatic detection-mechanism explains why we cannot tickle ourselves (Bays et al., 2006), why we perceive a stable visual world despite our frequent quick, or saccadic, eye movements (Sommer and Wurtz, 2006) and is reflected in different brain activation patterns between self-generated and exogenous visual stimulation (Matsuzawa et al., 2005). It is thought that the detection is accomplished via an efference copy (or corollary discharge) of the motor command which is compared to incoming afferent signals to distinguish re-afference from ex-afference. Such a differentiation has been implied to demonstrate causal reasoning in rats (Blaisdell et al., 2006; Clayton and Dickinson, 2006; Waldmann et al., 2006). Even robots can use such "self-modeling" to generate a continuously updated model of themselves and their environment (Bongard et al., 2006).

At the same time, by controlling the environmental input using operant feedback loops, individuals exert their effect not only on themselves, but their survival and procreation in the environment they shape for themselves directly affects evolution. This has been shown in the field, e.g., for western bluebirds, which dissociate into different niches according to their level of aggression (Duckworth, 2006). In humans such mechanisms have been proposed to explain otherwise hard to understand phenomena such as high IQ heritability estimates and associated paradoxes (i.e., increasing IQ heritability with age/experience and the "Flynn-Effect" of increasing IQ over generations) (Dickens and Flynn, 2001; Toga and Thompson, 2005). Another good example is the evolution of brain size. Most inter- and intraspecific interactions can be conceptualized as pursuit/evasion contests (e.g. predator/prey, male/female, dominant/subordinate etc.). There are two reports on such contests leading to increased brain size. The first details how small-brained prey are more likely to be caught by predators, presumably because their capacity for behavioral variability is also smaller (Shultz and Dunbar, 2006). The second shows that the largest relative brain sizes among primate species are associated with monogamous mating systems, raising the suspicion that unpredictable mating strategies are the most successful ones in monogamous species (Schillaci, 2006). Other research in birds ties the evolution of brain size both to behavioral variability and migration: birds with larger brains are both more likely to be sedentary and cope better in novel environments. The hypothesis here is that a sedentary lifestyle in seasonally changing habitats requires significant behavioral flexibility. Operant feedback provides flexible birds with more resources which enable them to support larger brains which in turn generate more behavioral variability: Brain size and behavioral flexibility co-evolved to out-compete other, smaller-brained birds which migrate in order to survive (Sol et al., 2005b; Sol et al., 2005a; Pravosudov et al., 2007). Thus, the interdependence of brain size, the level of behavioral variability it provides and the energy supply by which it is constrained are starting to unravel.

However, despite all these insights into the ultimate causes of the operant loop, until very recently, its proximate causes, the underlying neurobiological mechanisms have remained largely elusive.

4.3. Research strategy

What are the neurobiological mechanisms by which brains accomplish operant processes? The standard experimental approach for most of the last 80 years has been to study vertebrates (mammals or birds) in operant conditioning chambers ("Skinner Box"). However, most of that research was carried out by animal psychologists and rarely addressed the biological substrate which subserves the complex behavioral processes described in the early psychological literature. Today, lesion studies in rats and transgenic mouse models are starting to yield some insights as to the brain regions potentially involved in operant processing (Corbit and Balleine, 2005; Everitt and Robbins, 2005; Yin et al., 2005; Yin and Knowlton, 2006; Lobo et al., 2007; Ostlund and Balleine, 2007) and first fMRI studies seem to indicate that homologous regions may be involved in humans as well (O'Doherty et al., 2004; Kim et al., 2006; Glascher et al., 2008; Tanaka et al., 2008).

However, even the studies using modern neuroscientific techniques share the same drawback with the more traditional, psychological experiments: The environment is not under the full control of the experimenter. In operant conditioning chambers, the animals always have to manipulate an object (the manipulandum), usually by pressing a lever, pushing a pole or pulling a chain, etc. Thus, the animals have the possibility of learning about the properties of the manipulandum rather then their own behavior. In other words, animals may learn that the depressed lever signals food in much the same way as Pavlov's dogs learned that the ring of the bell signals food. But how can one get rid of the manipulandum and still operantly train a meaningful behavior?

Skinner was very close to the solution with his 'superstition' experiments (Skinner, 1947). He randomly dropped food pellets in a chamber with an individual pigeon. Whatever behavior the animals were performing at the time of food delivery was reinforced and thus increased in frequency. However, even in this experiment, the animals' stimulus situation was not kept constant. For instance, if the animal would rotate to look at the other end of the chamber, it might have associated either the other end of the chamber or the visual motion stimuli with the reward, and not its own behavior. To properly separate classical (relationships in the environment) from operant processes (consequences of one's own behavior) it is required to have control over the stimulus situation to such an extent, that the environmental stimuli can be switched on or off at the design of the experimenter at any time during the experiment. To this day, none of the vertebrate experimental situations offer this degree of control.

Invertebrate model systems offer an easier inroad into this challenging task. Some invertebrates have a comparatively limited sensory repertoire and for these animals the number of stimuli to be controlled is already lower than for vertebrates. Their brains are usually less complex and one can experimentally interfere with their sensory function more easily, or their anatomy lends itself to removing entire sensory organs. Some invertebrates, e.g., gastropods, offer complete stimulus control by using semi-intact preparations or brain explants. For other invertebrates, e.g., insects, elaborate technical setups exist which allow superb stimulus control even in the intact animal. The marine snail *Aplysia* offers in vitro operant conditioning in a numerically less complex brain which is easily amenable to physiological experimentation. One

of the model systems with the most sophisticated genetic toolbox, the fruit fly *Drosophila,* can be tethered such that its behavior can be monitored while neutral as well as biologically relevant stimuli can be applied automatically without the need to handle the animal. Thus, by using complementing invertebrate models systems, one can study operant processes on the single cell, network and behavioral level using rigorous behavioral experiments as well as advanced genetic and physiological manipulations. Given the high homology in classical learning processes and the ubiquitous nature of operant processes in all animals (see above), one would expect that the basic biological functional principles underlying operant behavior and learning will be conserved as well.

In the period covered by this habilitation, I have used the existing model systems to develop the experimental designs to study how spontaneous behavior is generated by the brain, how ongoing behavior is modified to control environmental stimuli (operant behavior) and how continued control of the environment can lead to lasting behavioral modifications (operant learning or habit formation).

The most challenging aspect so far has been how spontaneous behavior is modified to control sensory input (operant behavior). None of the genetic screens or manipulations in flies or snails so far have yielded any insight into the biological substrate of operant behavior. Therefore, my strategy is to study how spontaneous behavior is generated and how operant behavior is transformed into operant memories by operant learning. Once we have made inroads into these processes, maybe we are better equipped for a more targeted approach on the mechanisms of operant behavior.

To study the generation of spontaneous behavior in flies we have adopted a mathematical tool which can distinguish a random series of events from a nonlinear series (Maye et al., 2007). Because flies show a nonlinear signature in the variability of all analyzed behaviors, this setup can now be used to screen flies with manipulated brain function for the brain areas involved in generating this spontaneous variability. A DFG-funded research project is currently being concluded which showed that it is feasible to optophysiologically record from all visible neurons in a isolated *Aplysia* buccal ganglia while thy are spontaneously generating behaviorally significant neural activity patterns.

The study of operant learning has the longest history and therefore we know more about this aspect of the operant process than of any other. The demarcation of operant learning from operant behavior was the starting point of my research endeavors (Wolf and Heisenberg, 1991). After first gathering insights into how the presence or absence stimuli under operant control influence the overall learning processes in my Diploma and PhD work (Brembs, 1996b, 2000; Brembs and Heisenberg, 2000, 2001), it was time to focus more sharply on the ‘pure’ operant learning without any contingent environmental stimuli (Brembs et al., 2002; Brembs et al., 2004). More recently, the genetic tools have finally arrived to use transgenic animals also in the demanding tethered *Drosophila* setup. The groundwork that has been laid in my Diploma and PhD work has now been put to good use in the last few years (Brembs and Wiener, 2006; Brembs et al., 2007; Brembs, 2008, in prep.; Brembs and Plendl, 2008, re-subm.). The results can be subsumed in my habilitation thesis.

4.4. Habilitation Thesis

The current literature and the data presented below are consistent with the hypothesis that most if not all brains share the common function of first generating spontaneous behavior and then evaluating re-afferent feedback from the environment to guide the generation of further actions (operant behavior and operant learning). This model of adaptive behavioral choice via operant processes relies on a non-linear mechanism generating behavioral variability (Maye et al., 2007) as a substrate for environmental feedback. The environmental feedback modifies the neurons involved in the non-linear mechanism using the reward/punishment circuits (Nargeot et al., 1999; Schwaerzel et al., 2003; Schultz, 2005; Brembs et al., 2007) and leads to a lasting change in the biophysical properties of the neurons in which operant behavior and reward/punishment converge (Brembs et al., 2002; Lorenzetti et al., 2006b). The molecular processes involved in bringing about these biophysical changes are distinct from the ones bringing about synaptic plasticity after classical learning (Lorenzetti et al., 2006a; Brembs and Plendl, 2008, resubm.). The experimental tools now exist to study how this operant form of learning interacts with other forms of learning, for instance classical learning (Brembs et al., 2004; Brembs, 2008, in prep.). First evidence points towards hierarchical interactions between these different memory systems which function to prevent premature habit formation of the operant system from interfering with generalization of classical memories (Brembs, 2008, in prep.).

5. List of publications submitted for the habilitation

1. Baier A.; Wittek B. and **Brembs B.*** (2002): *Drosophila* as a new model organism for the neurobiology of aggression? J. Exp. Biol. *205*, 1233-1240
2. **Brembs B.**; Lorenzetti F.D.; Reyes F.D.; Baxter D.A. and Byrne J.H. (2002): Operant Reward Learning in *Aplysia*: Neuronal Correlates and Mechanisms. Science *296*, 1706-1709
3. **Brembs B.***; Baxter D.A. and Byrne J.H. (2004): Extending *in vitro* conditioning in *Aplysia* to analyze operant and classical processes in the same preparation. Learn. Mem. *11*, 412-420
4. Phillips A.M.; Smart R.; Strauss R.; **Brembs B.** and Kelly, L.E. (2005): The *Drosophila black* enigma: the molecular and behavioural characterization of the *black*^{1} mutant allele. Gene *351C*, 131-142
5. **Brembs B.*** and Wiener, J. (2006): Context generalization and occasion setting in *Drosophila* visual learning. Learn. Mem. *13*, 618-628
6. **Brembs, B.*** and Hempel de Ibarra, N. (2006): Different parameters support discrimination and generalization in *Drosophila* at the flight simulator. Learn. Mem. *13*, 629-637
7. Maye, A.; Hsieh, C.; Sugihara, G. and **Brembs, B.*** (2007): Order in spontaneous behavior. PLoS One *2*: e443
8. **Brembs, B.**; Christiansen, F.; Pflüger, H.J. and Duch, C. (2007): Flight initiation and maintenance deficits in flies with genetically altered biogenic amine levels. J. Neurosci. *27*, 11122-11131
9. **Brembs, B.*** (2008): Operant learning of *Drosophila* at the torque meter. JoVE *16*. http://www.jove.com/index/Details.stp?ID=731, doi: 10.3791/731
10. **Brembs, B.*** and Plendl, W. (2008): Double dissociation of protein-kinase C and adenylyl cyclase manipulations on operant and classical learning in *Drosophila*. Current Biology. *Re-subm.*
11. **Brembs, B.*** (2008): Mushroom-bodies regulate habit formation in *Drosophila*. J. Neurosci. *Subm*.
12. **Brembs B**.* (2008): The importance of being active. J. Neurogen. *Subm.*

* corresponding author

6. Discussion

The study of the biological mechanisms of spontaneous behavioral variability is only in its infancy. Our evidence that a nonlinear mechanism may be involved in producing the variability in flight behavior has only scratched the surface of this topic (Maye et al., 2007). While our data seem to conform well with other fly studies (Martin et al., 1999; Martin et al., 2001), the research in other animals is not yet far enough to draw any firm conclusions about the evolutionary conservation of these mechanisms. A first mechanistic approach has been published in the leech isolated nervous system, but also there much more research is required for a full understanding (Briggman et al., 2005). A computational analysis in the lobster stomatogastric nervous system may be used to argue that degeneracy in the nervous system is one such nonlinear mechanism responsible for variations in behavior (Prinz et al., 2004), but the link is rather tenuous and indirect. Thus, as of this writing, there are now tools available to start to unravel how brains manage to constantly vary ongoing behavior and to seemingly randomly choose between different behavioral options, but an understanding of these mechanisms is still far in the future.

There is a lot more data on the mechanisms of operant learning and how they interact with other learning processes such as classical learning. Much as classical learning involves neuronal modifications in the sensory pathways, the evidence points towards operant learning involving changes in the circuits involved in motor control (Corbit et al., 2001; Brembs et al., 2002; Brembs, 2003b; Mozzachiodi et al., 2003; Lorenzetti et al., 2006b; Ostlund and Balleine, 2007). However, while in other forms of learning the unifying principle appears to be synaptic plasticity, there is not sufficient evidence in operant learning as to whether there is a common process. The only currently known mechanism involves neuronal rather than synaptic plasticity (Brembs et al., 2002; Brembs, 2003b, a). The distinctiveness of operant learning is continued on the genetic level where no crosstalk was detected between the genetic networks underlying operant learning and those underlying other forms of learning (Brembs and Plendl, 2008, re-subm.). Interestingly, in vertebrates, the pathway our PKCi experiments interfered with eventually leads to the activation of dopamine and cyclic adenosine 3′,5′-monophosphate-regulated phosphoprotein, 32 kDa – DARPP-32, which is involved in a variety of processes and disorders associated with operant functioning (Greengard et al., 1999; Greengard, 2001; Svenningson and Greengard, 2006). The research implies that the acquisition of skills and habits, such as writing, driving a car, tying laces or our going to bed rituals is not only processed by different brain structures than our explicit memories, the neurons also use different biochemical processes to store these memories. If these early results were substantiated, classical conditioning paradigms cannot serve as the general tools for all learning and memory research as they do today. Further research in this area is required to elucidate the molecular processes during and after operant learning as well as the brain areas involved in operant learning in the fly.

As the mechanisms of other forms of learning become increasingly understood, more and more experiments are being directed towards the interactions of multiple memory systems. The evidence in flies points towards analogous interactions between operant and classical memory systems in insects and

mammals: in ethologically relevant learning situations, (i.e., situations in which the animal's behavior controls both initially neutral sensory stimuli as well as biologically relevant ones) a hierarchical, reciprocal interaction inhibits operant learning and facilitates learning about the predictive (classical) stimuli (Brembs, 2008, in prep.). The facilitation of classical learning has been observed in virtually every animal ever tested: humans (James, 1890; Slamecka and Graf, 1978), monkeys (Kornell and Terrace, 2007), cats (Thorndike, 1898), rats (Blaisdell et al., 2006) and even flies (Brembs and Heisenberg, 2000; Brembs and Wiener, 2006). The inhibition of operant learning has directly been observed only in flies and serves to prevent premature habit formation which would interfere with generalization of the classical memory (Brembs, 2008, in prep.). Vertebrate data on habit formation can be interpreted to conform to such an organization as well (Yin and Knowlton, 2006). The picture emerges that ethologically relevant learning situations consist of biologically disparate learning systems or modules which interact to accomplish adaptive behavior. In this picture, spontaneous behavioral variability can be seen as the starting point which not only directly guarantees survival by making behavior more difficult to predict for predators, prey, competitors or mates, but conveys additional fitness benefits by contributing critically to the operant processes which provides every animal with predictive knowledge about its environment and the consequences of behaving in it.

6.1. The broader scope of operant research

Considering what we know today, it may not be so surprising that the evolutionary relevance of spontaneous behavioral variability and operant learning reverberates in human psychology. A host of psychiatric disorders is associated with the operant loop and the capability of controlling the environment it confers. For example, patients with depression often report that they have lost control of their lives. Interestingly, "Learned Helplessness" is a standard animal model for depression in which animals develop symptoms of depression by exposure to uncontrollable shocks (Seligman, 1975; Maier and Watkins, 2005). The degree of control over such stressors is critical for the development of depression (Amat et al., 2005). Such operant control is even said to slow the cognitive decay occurring in patients when they enter the late stage of Amyotrophic Lateral Sclerosis (ALS, Lou Gehrig's disease), a degenerative motorneuron disorder (Birbaumer, 2006). Anorexic patients often report that controlling their eating and hunger is the only means of control left in their lives. Often these patients, when they eat, cut the food into always the same number of pieces and chew them for the same number of times. Anorexia nervosa and obsessive compulsive disorder share this symptom of rituals/stereotypies and show a high degree of comorbidity (Steinglass and Walsh, 2006).

The experience of willing to do something and then successfully doing it is absolutely central to developing a sense of agency, i.e., of who we are (and who we are not) and that we are in control (and not being controlled). Early childhood abuse and the feeling of utter lack of control it entails can severely compromise the development of this sense. A recurrent pattern in patients with borderline personality disorder is childhood abuse (i.e., uncontrollable aversive stimulation) and later self-mutilation. Frequently these self-harming

patients report as a reason for the self-harm that they need to assure themselves that the body they injure is actually theirs and that they have control over it. Apparently, the damage done to their sense of self is so severe that strong, painful feedback is required to re-initiate it. Self-mutilation and these so-called dissociation experiences show a high co-morbidity, irrespective of the disorder the patients suffer from (Brodsky et al., 1995). For instance, dissociation is also reported from patients with dissociative identity disorder, alien hand syndrome, or schizophrenic delusions (Bays et al., 2006).

Most often, these disorders are associated with alterations in the activity of the midbrain dopamine neurons which are thought to mediate reward (Schultz and Dickinson, 2000; Schultz, 2001, 2002, 2005). This insight ties, e.g., Parkinson and schizophrenia also tightly to operant models (Drew et al., 2007). Parkinson patients are impaired in operant learning (Knowlton et al., 1996). The most common treatment of Parkinson's disease is administering the dopamine precursor L-DOPA. Schizophrenics are treated with a group of antipsychotics, most of which target and inhibit the D_2 dopamine receptor. Some of these antipsychotic drugs have Parkinson-like side-effects. Interestingly, L-DOPA and the antipsychotic haloperidol have opposite effects on operant decision-making in humans (Pessiglione et al., 2006). Maybe not surprisingly, the dopaminergic system is the common structure involved in all of the abovementioned disorders and the crucial point of reference according to which the balance of stereotypy or variability, hyperacivity and passivity, motivation or lack of interest appears to be tared (Kaplan and Oudeyer, 2007).

6.2. Outlook: Invertebrate neuroscience in the post-genomic era

In insects, the biogenic amine octopamine appears to mediate reward in a similar manner as dopamine in vertebrates and mollusks (Hammer, 1997; Schwaerzel et al., 2003; Riemensperger et al., 2005). Also paralleling the actions of the dopaminergic systems in vertebrates, octopamine as the main mediator of reward is also involved in controlling movements in insects. This is accomplished by a set of homologous octopaminergic neurons (DUM/VUM neurons) mediating reward in the brain and controlling behavior in the rest of the body (Roeder, 2005; Brembs et al., 2007). Aggression is another trait where this striking analogy can be observed. While other amines are involved in aggression (de Almeida et al., 2005; Phillips et al., 2005), in mammals as well as in insects, dopamine/octopamine plays an important role in the initiation of aggressive behaviors (Baier et al., 2002; de Almeida et al., 2005; Hoyer et al., 2008). Are these findings a mere coincidence or evidence that systems mediating primary rewards have co-evolved with those mediating behavior initiation and control precisely because of the rewarding properties of controlling the environment with behavior? Obviously, understanding the neural bases of operant behavior and learning is not only an important academic question, but also very much a mental health one.

The current relative paucity of mechanistical knowledge in operant learning stems in part from research into operant learning being conceptually much more challenging than, e.g., classical conditioning. However, recent progress in invertebrate neuroscience suggests that the now classic Kandelian approach of relying heavily on simpler brains while developing tools and models for ver-

tebrate research is even more promising today in the age of advanced molecular, genetic, imaging and physiological repertoires in invertebrates than 30 years ago (Greenspan, 2005; Menzel et al., 2006). Even in the post-genomic era, invertebrate models offer the possibility to rapidly and effectively learn about important principles and molecules which can then be used to reduce the complexity of the vertebrate brain (Brembs, 2003b). Besides offering a more effective avenue into studying the neural basis of operant conditioning, such an integrative approach will provide us with insights into the exciting question of why invertebrate and vertebrate brains are structurally so very different even though the basic demands of life are quite similar in both groups. Moreover, a multi-faceted approach will allow us to distinguish general mechanisms from species-specific adaptations. Coincidentally, using multiple model systems effectively reduces the number of vertebrate experimental animals, working towards the more and more widely discussed '3R' goals — refinement, reduction and replacement (Axton, 2006). Combining the rapid technical advancements also in vertebrate physiology, imaging and behavior (Kleinfeld and Griesbeck, 2005) with modern computational power, neuroscience is now more than ready to finally tackle the neurobiology of operant learning on a broad scale.

7. References

Amat J, Baratta MV, Paul E, Bland ST, Watkins LR, Maier SF (2005) Medial prefrontal cortex determines how stressor controllability affects behavior and dorsal raphe nucleus. Nat Neurosci 8:365-371.

Axton M (2006) Animal research and the search for understanding. Nat Genet 38:497-498.

Baier A, Wittek B, Brembs B (2002) Drosophila as a new model organism for the neurobiology of aggression? J Exp Biol 205:1233-1240.

Bays PM, Flanagan JR, Wolpert DM (2006) Attenuation of Self-Generated Tactile Sensations Is Predictive, not Postdictive. PLoS Biology 4:e28.

Belanger JH, Willis MA (1996) Adaptive control of odor-guided locomotion: Behavioral flexibility as an antidote to environmental unpredictability. Adaptive Behavior 4:217-253.

Birbaumer N (2006) Breaking the silence: Brain-computer interfaces (BCI) for communication and motor control. Psychophysiology 43:517-532.

Blaisdell AP, Sawa K, Leising KJ, Waldmann MR (2006) Causal Reasoning in Rats. Science 311:1020-1022.

Bongard J, Zykov V, Lipson H (2006) Resilient Machines Through Continuous Self-Modeling. Science 314:1118-1121.

Brembs B (1996a) Chaos, cheating and cooperation: Potential solutions to the Prisoner's Dilemma. Oikos 76:14-24.

Brembs B (1996b) Classical and Operant Conditioning in *Drosophila* at the Flight Simulator. In: Department of Genetics, p 30. Würzburg: Julius-Maximilians-Universität.

Brembs B (2000) An analysis of associative conditioning in *Drosophila* at the flight simulator. In: Department of Genetics, p 41. Würzburg: University of Würzburg.

Brembs B (2003a) Operant reward learning in Aplysia. Current Directions in Psychological Science 12:218-221.

Brembs B (2003b) Operant conditioning in invertebrates. Current Opinion in Neurobiology 13:710-717.

Brembs B (2008) Operant learning of *Drosophila* at the torque meter. JoVE 16.:http://www.jove.com/index/Details.stp?ID=731, doi: 710.3791/3731.

Brembs B (2008, in prep.) Mushroom-bodies regulate habit-formation in *Drosophila*. J Neurosci.

Brembs B (2008, subm.) The importance of being active. J Neurogenet spec. issue.

Brembs B, Heisenberg M (2000) The operant and the classical in conditioned orientation in *Drosophila melanogaster* at the flight simulator. Learn Mem 7:104-115.

Brembs B, Heisenberg M (2001) Conditioning with compound stimuli in *Drosophila melanogaster* in the flight simulator. J Exp Biol 204:2849-2859.

Brembs B, Wiener J (2006) Context generalization and occasion setting in *Drosophila* visual learning. Learn Mem 13:618-628.

Brembs B, Hempel de Ibarra N (2006) Different parameters support generalization and discrimination learning in *Drosophila* at the flight simulator. Learn Mem 13:629-637.

Brembs B, Plendl W (2008, re-subm.) Double dissociation of protein-kinase C and adenylyl cyclase manipulations on operant and classical learning in Drosophila. Curr Biol.

Brembs B, Baxter DA, Byrne JH (2004) Extending *in vitro* conditioning in *Aplysia* to analyze operant and classical processes in the same preparation. Learning and Memory 11:412-420.

Brembs B, Christiansen F, Pfluger HJ, Duch C (2007) Flight Initiation and Maintenance Deficits in Flies with Genetically Altered Biogenic Amine Levels. J Neurosci 27:11122-11131.

Brembs B, Lorenzetti FD, Reyes FD, Baxter DA, Byrne JH (2002) Operant reward learning in Aplysia: neuronal correlates and mechanisms. Science 296:1706-1709.

Briggman KL, Abarbanel HD, Kristan WB, Jr. (2005) Optical imaging of neuronal populations during decision-making. Science 307:896-901.

Brodsky BS, Cloitre M, Dulit RA (1995) Relationship of dissociation to self-mutilation and childhood abuse in borderline personality disorder. Am J Psychiatry 152:1788-1792.

Churchland MM, Afshar A, Shenoy KV (2006) A Central Source of Movement Variability. Neuron 52:1085-1096.

Clayton N, Dickinson A (2006) Rational rats. Nat Neurosci 9:472-474.

Corbit LH, Balleine BW (2005) Double dissociation of basolateral and central amygdala lesions on the general and outcome-specific forms of pavlovian-instrumental transfer. J Neurosci 25:962-970.

Corbit LH, Muir JL, Balleine BW (2001) The role of the nucleus accumbens in instrumental conditioning: Evidence of a functional dissociation between accumbens core and shell. J Neurosci 21:3251-3260.

Dayan P, Niv Y, Seymour B, Daw ND (2006) The misbehavior of value and the discipline of the will. Neural Networks 19:1153-1160.

de Almeida RMM, Ferrari PF, Parmigiani S, Miczek KA (2005) Escalated aggressive behavior: Dopamine, serotonin and GABA. Eur J Pharmacol 526:51-64.

Dickens WT, Flynn JR (2001) Heritability estimates versus large environmental effects: the IQ paradox resolved. Psychol Rev 108:346-369.

Donahoe JW, Palmer DC, Burgos JE (1997) The S-R issue: Its status in behavior analysis and in Donhaoe and Palmer's "Learning and Complex Behavior" (with commentaries and reply). J Exp Anal Behav 67:193-273.

Drew MR, Simpson EH, Kellendonk C, Herzberg WG, Lipatova O, Fairhurst S, Kandel ER, Malapani C, Balsam PD (2007) Transient Overexpression of Striatal D2 Receptors Impairs Operant Motivation and Interval Timing. J Neurosci 27:7731-7739.

Driver PM, Humphries N (1988) Protean behavior: The biology of unpredictability. Oxford, England: Oxford University Press.

Duckworth R (2006) Aggressive behaviour affects selection on morphology by influencing settlement patterns in a passerine bird. Proc R Soc Lond B:FirstCite.

Everitt BJ, Robbins TW (2005) Neural systems of reinforcement for drug addiction: from actions to habits to compulsion. Nat Neurosci 8:1481-1489.

Fox MD, Snyder AZ, Vincent JL, Raichle ME (2007) Intrinsic Fluctuations within Cortical Systems Account for Intertrial Variability in Human Behavior. Neuron 56:171-184.

Friston KJ, Tononi G, Reeke GN, Jr., Sporns O, Edelman GM (1994) Value-dependent selection in the brain: simulation in a synthetic neural model. Neuroscience 59:229-243.

Glascher J, Hampton AN, O'Doherty JP (2008) Determining a Role for Ventromedial Prefrontal Cortex in Encoding Action-Based Value Signals During Reward-Related Decision Making. Cereb Cortex.

Glimcher P (2003) Decisions, uncertainty, and the brain: the science of neuroeconomics. Cambridge, MA: MIT.

Glimcher PW (2005) Indeterminacy in brain and behavior. Annu Rev Psychol 56:25-56.

Gormezano I, Tait RW (1976) The Pavlovian analysis of instrumental conditioning. Pavlov J Biol Sci 11:37-55.

Greengard P (2001) The Neurobiology of Slow Synaptic Transmission. Science 294:1024-1030.
Greengard P, Allen PB, Nairn AC (1999) Beyond the Dopamine Receptor: the DARPP-32/Protein Phosphatase-1 Cascade. Neuron 23:435-447.
Greenspan RJ (2005) No Critter Left Behind: An Invertebrate Renaissance. Curr Biol 15:R671-R672.
Grobstein P (1994) Variability in behavior and the nervous system. In: Encyclopedia of Human Behavior. (Ramachandran VS, ed), pp 447-458. New York: Academic Press.
Guthrie ER (1952) The Psychology of Learning. New York: Harper.
Hammer M (1997) The neural basis of associative reward learning in honeybees. Trends Neurosci 20:245-252.
Hebb DO (1956) The Distinction between Classical and Instrumental. Can J Psychol 10:165-166.
Heisenberg M (1983) Initiale Aktivität und Willkürverhalten bei Tieren. Naturwissenschaften:70-78.
Heisenberg M (1994) Voluntariness (Willkürfähigkeit) and the general organization of behavior. L Sci Res Rep **55**:147-156.
Heisenberg M, Wolf R, Brembs B (2001) Flexibility in a single behavioral variable of Drosophila. Learn Mem 8:1-10.
Hoyer SC, Eckart A, Herrel A, Zars T, Fischer SA, Hardie SL, Heisenberg M (2008) Octopamine in Male Aggression of Drosophila. Curr Biol 18:159-167.
Jablonski PG, Strausfeld NJ (2000) Exploitation of an ancient escape circuit by an avian predator: prey sensitivity to model predator display in the field. Brain Behav Evol 56:94-106.
Jablonski PG, Strausfeld NJ (2001) Exploitation of an ancient escape circuit by an avian predator: relationships between taxon-specific prey escape circuits and the sensitivity to visual cues from the predator. Brain Behav Evol 58:218-240.
James W (1890) The Principles of Psychology. New York: Holt.
Kaplan F, Oudeyer P-Y (2007) In search of the neural circuits of intrinsic motivation Frontiers in Neuroscience 1:225-236.
Kim H, Shimojo S, O'Doherty JP (2006) Is Avoiding an Aversive Outcome Rewarding? Neural Substrates of Avoidance Learning in the Human Brain. PLoS Biology 4:e233.
Kimmel H (1965) Instrumental inhibitory factors in classical conditioning. In: Classical Conditioning (WF P, ed). New York: Appleton-Century-Crofts.
Kleinfeld D, Griesbeck O (2005) From Art to Engineering? The Rise of In Vivo Mammalian Electrophysiology via Genetically Targeted Labeling and Nonlinear Imaging. PLoS Biology 3:e355.
Knowlton BJ, Mangels JA, Squire LR (1996) A neostriatal habit learning system in humans. Science 273:1399-1402.
Konorski J, Miller S (1937a) Further remarks on two types of conditioned reflex. J Gen Psychol 17:405-407.
Konorski J, Miller S (1937b) On two types of conditioned reflex. J Gen Psychol 16:264-272.
Kornell N, Terrace HS (2007) The Generation Effect in Monkeys. Psychol Sci 18:682-685.
Lobo MK, Cui Y, Ostlund SB, Balleine BW, William Yang X (2007) Genetic control of instrumental conditioning by striatopallidal neuron-specific S1P receptor Gpr6. Nat Neurosci 10:1395-1397.

Lorenzetti FD, Baxter DA, Byrne JH (2006a) Both PKA and PKC are necessary for plasticity in a single-cell analogue of operant conditioning. In: Annual meeting of the Society for Neuroscience. Atlanta, Ga. USA.

Lorenzetti FD, Mozzachiodi R, Baxter DA, Byrne JH (2006b) Classical and operant conditioning differentially modify the intrinsic properties of an identified neuron. Nat Neurosci 9:17-29.

Maier SF, Watkins LR (2005) Stressor controllability and learned helplessness: The roles of the dorsal raphe nucleus, serotonin, and corticotropin-releasing factor. Neurosci Biobehav Rev 29:829-841.

Martin JR, Ernst R, Heisenberg M (1999) Temporal pattern of locomotor activity in Drosophila melanogaster. Journal of Comparative Physiology A Sensory Neural and Behavioral Physiology 184:73-84.

Martin JR, Faure P, Ernst R (2001) The power law distribution for walking-time intervals correlates with the ellipsoid-body in Drosophila. J Neurogenet 15:205-219.

Matsuzawa M, Matsuo K, Sugio T, Kato C, Nakai T (2005) Temporal relationship between action and visual outcome modulates brain activation: an fMRI study. Magn Reson Med Sci 4:115-121.

Mauk MD (2000) The potential effectiveness of simulations versus phenomenological models. Nat Neurosci 3:649-651.

Maye A, Hsie C-h, Sugihara G, Brembs B (2007) Order in spontaneous behavior. PLoS One 2:e443.

McNamara JM, Barta Z, Houston AI (2004) Variation in behaviour promotes cooperation in the Prisoner's Dilemma game. Nature 428:745-748.

Menzel R, Leboulle G, Eisenhardt D (2006) Small Brains, Bright Minds. Cell 124:237-239.

Miller GF (1997) Protean primates: The evolution of adaptive unpredictability in competition and courtship. In: Machiavellian Intelligence II: Extensions and evaluations (Whiten A, Byrne RW, eds), pp 312-340. Cambridge: Cambridge University Press.

Miller S, Konorski J (1928) Sur une forme particuliere des reflexes conditionnels. C r Soc Biol 99:1155-1157.

Miller S, Konorski J (1969) On a Particular Form of Conditioned Reflex. J Exp Anal Behav 12:187-189.

Mozzachiodi R, Lechner H, Baxter D, Byrne J (2003) An *in vitro* analogue of classical conditioning of feeding in *Aplysia*. Learning and Memory 10:478-494.

Nargeot R, Baxter DA, Patterson GW, Byrne JH (1999) Dopaminergic synapses mediate neuronal changes in an analogue of operant conditioning. J Neurophysiol 81:1983-1987.

Neuringer A (2004) Reinforced variability in animals and people: implications for adaptive action. Am Psychol 59:891-906.

O'Doherty J, Dayan P, Schultz J, Deichmann R, Friston K, Dolan RJ (2004) Dissociable roles of ventral and dorsal striatum in instrumental conditioning. Science 304:452-454.

Okuyama T (2007) Individual behavioral variation in predator–prey models. Ecological Research 10.1007/s11284-007-0425-5.

Ostlund SB, Balleine BW (2007) Orbitofrontal cortex mediates outcome encoding in pavlovian but not instrumental conditioning. J Neurosci 27:4819-4825.

Park JS, Onodera T, Nishimura S, Thompson RF, Itohara S (2006) Molecular evidence for two-stage learning and partial laterality in eyeblink conditioning of mice. Proc Natl Acad Sci USA 103:5549-5554.

Pessiglione M, Seymour B, Flandin G, Dolan RJ, Frith CD (2006) Dopamine-dependent prediction errors underpin reward-seeking behaviour in humans. 442:1042-1045.
Phillips AM, Smart R, Strauss R, Brembs B, Kelly LE (2005) The Drosophila black enigma: the molecular and behavioural characterization of the black1 mutant allele. Gene 351:131-142.
Platt ML (2004) Unpredictable primates and prefrontal cortex. Nat Neurosci 7:319-320.
Plotnik JM, de Waal FBM, Reiss D (2006) Self-recognition in an Asian elephant. PNAS 103:17053-17057.
Pravosudov VV, Sanford K, Hahn TP (2007) On the evolution of brain size in relation to migratory behaviour in birds. Animal Behaviour 73:535-539.
Prinz AA, Bucher D, Marder E (2004) Similar network activity from disparate circuit parameters. Nat Neurosci 7:1345-1352.
Prokasy W (1965) Classical eyelid conditioning: Experimenter operations, task demands and response shaping. In: Classical Conditioning (Prokasy W, ed). New York: Appleton-Century-Crofts.
Reiss D, Marino L (2001) Mirror self-recognition in the bottlenose dolphin: A case of cognitive convergence. PNAS 98:5937-5942.
Rescorla RA (1987) A Pavlovian analysis of goal-directed behavior. Am Psychol 42:119-129.
Rescorla RA, Solomon RL (1967) Two-process learning theory: Relationships between Pavlovian conditioning and instrumental learning. Psychol Rev 74:151-182.
Riemensperger T, Voller T, Stock P, Buchner E, Fiala A (2005) Punishment Prediction by Dopaminergic Neurons in Drosophila. Curr Biol 15:1953-1960.
Roeder T (2005) Tyramine and octopamine: ruling behavior and metabolism. Annu Rev Entomol 50:447-477.
Schillaci MA (2006) Sexual Selection and the Evolution of Brain Size in Primates. PLoS ONE 1:e62.
Schultz W (2001) Reward signaling by dopamine neurons. Neuroscientist 7:293-302.
Schultz W (2002) Getting formal with dopamine and reward. Neuron 36:241-263.
Schultz W (2005) Behavioral Theories and the Neurophysiology of Reward. Annu Rev Psychol.
Schultz W, Dickinson A (2000) Neuronal coding of prediction errors. Annu Rev Neurosci 23:473-500.
Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelin F, Birman S, Heisenberg M (2003) Dopamine and Octopamine Differentiate between Aversive and Appetitive Olfactory Memories in *Drosophila*. J Neurosci 23:10495-10502.
Seligman M (1975) Helplessness: On depression, development, and death.: W. H. Freeman.
Shultz S, Dunbar R (2006) Chimpanzee and felid diet composition is influenced by prey brain size. Biology Letters 2:505-508.
Skinner B (1947) 'Superstition' in the pigeon. J Exp Psychol 38:168-172.
Skinner BF (1935) Two types of conditioned reflex and a pseudo type. J Gen Psychol 12:66-77.
Skinner BF (1937) Two types of conditioned reflex: A reply to Konorski and Miller. J Gen Psychol 16:272-279.
Skinner BF (1938) The behavior of organisms. New York: Appleton.
Slamecka NJ, Graf P (1978) Generation Effect - Delineation of a Phenomenon. J Exp Psychol [Hum Learn] 4:592-604.

Sol D, Lefebvre L, Rodriguez-Teijeiro J (2005a) Brain size, innovative propensity and migratory behaviour in temperate Palaearctic birds. Proceedings of the Royal Society B: Biological Sciences 272:1433-1441.

Sol D, Duncan RP, Blackburn TM, Cassey P, Lefebvre L (2005b) Big brains, enhanced cognition, and response of birds to novel environments. Proc Natl Acad Sci USA 102:5460-5465.

Sommer MA, Wurtz RH (2006) Influence of the thalamus on spatial visual processing in frontal cortex. Nature advanced online publication.

Steinglass J, Walsh BT (2006) Habit learning and anorexia nervosa: A cognitive neuroscience hypothesis. Int J Eat Disord.

Svenningson P, Greengard P (2006) A master regulator in the brain. The Scientist 20:40.

Tanaka SC, Balleine BW, O'Doherty JP (2008) Calculating consequences: brain systems that encode the causal effects of actions. J Neurosci 28:6750-6755.

Thorndike E (1898) Animal Intelligence. An Experimental Study of the Associative Processes in Animals. New York: Macmillan.

Thorndike EL (1911) Animal Intelligence. New York: Macmillan.

Todorov E (2004) Optimality principles in sensorimotor control. Nat Neurosci 7:907-915.

Toga AW, Thompson PM (2005) Genetics of brain structure and intelligence. Annu Rev Neurosci 28:1-23.

Trapold MA, Overmier JB (1972) The second learning process in instrumental conditioning. In: Classical Conditioning II: Current research and theory (Black AH, Prokasy WF, eds), pp 427-452. New York: Appleton-Century-Crofts.

von Holst E, Mittelstaedt H (1950) Das Reafferenzprinzip. Wechselwirkungen zwischen Zentralnervensystem und Peripherie. Naturwissenschaften:464-476.

Waldmann MR, Hagmayer Y, Blaisdell AP (2006) Beyond the Information Given: Causal Models in Learning and Reasoning. Current Directions in Psychological Science 15:307-311.

Watson J (1928) The ways of behaviorism. New York: Harper & Brothers Pub.

Webb B (2004) Neural mechanisms for prediction: do insects have forward models? Trends Neurosci 27:278-282.

Wegner DM (2002) The illusion of conscious will. Boston: Bradford Books/MIT press.

Wolf R, Heisenberg M (1991) Basic organization of operant behavior as revealed in *Drosophila* flight orientation. J Comp Physiol [A] 169:699-705.

Yin HH, Knowlton BJ (2006) The role of the basal ganglia in habit formation. Nat Rev Neurosci 7:464-476.

Yin HH, Ostlund SB, Knowlton BJ, Balleine BW (2005) The role of the dorsomedial striatum in instrumental conditioning. Eur J Neurosci 22:513-523.

8. Acknowledgments

First and foremost I would like to thank my mentors Drs. Heisenberg, Byrne and Menzel. Their tireless teachings, advice, support, encouragement and insight have been invaluable. Without them, I would be a different person and this habilitation would not have happened. They all must have used most of their toner money for my letters of recommendation.

Jochen Pflüger deserves a very special mention and emphasis because he bailed me out and offered me his postdoctoral position to survive when I needed it the most. Without him, I would probably be flipping burgers now.

I am also especially indebted to my colleagues and co-authors. Reinhard Wolf is a dear friend and a never ending source of information, enlightenment, knowledge, wisdom and funny emails. Alexander Maye who tirelessly checks if the algorithms I send him could be useful for us. Doug Baxter always manages to provide some crucial improvements for any manuscript. Fred Lorenzetti who by now must be able to pick out B51 blindfolded at 4am, wearing mittens and without microscope. Riccardo Mozzachiodi had the unfathomable patience of standing out to share not only a 3 square meter lab with me for over 3 years, but also my flat for more than a month after Houston was flooded. Britta Witt(ek) with whom it was easy to keep a close friendship over many years even with only loose contact. Roland Strauss who will most likely succeed Dr. Heisenberg as chair in Würzburg, I hope, and whose smart and witty mind seems to never to stop. Jan Wiener was my first undergraduate diploma student (even though he was Heisenberg's). Natalie Hempel de Ibarra not only figured out what on earth the flies are seeing as green and blue but also provided a constant flow of support, encouragement, energy and warmth. Frauke Christiansen did great work with not one but two advisors. I hold Carsten Duch in high esteem not the least for his never ending humor, advice and patience, particularly when I was being slow on insect flight. George Sugihara, the famous man, provided me with a brief but very enlightening glimpse into the fascinating world of nonlinear dynamics. Bill Kristan and his lab have been exceptionally hospitable hosting me on several occasions to try *Aplysia* imaging and Quentin Gaudry there bore the brunt of the work. I will publish an imaging paper in *Aplysia* and you will be an author, I promise. Betsy Cropper who is always so kind and soft-spoken in one of the best labs I know. Together with Klaude Weiss they have supported me, hosted me more than once and given me the chance to learn so much from them. I can only hope that this habilitation will provide me with an opportunity to pay some of your kindness back. Without Wolfgang Plendl's intuition and ingenious ideas, the genetic breakthrough in operant learning would not have been possible. Bernard Balleine still is my main source of knowledge for all operant things rodent and a great guy all around. Peter Dayan's sharp mind is always good for stimulating input, challenging questions and critique – the way it should be. David Glanzman's bull-fight analogy provided a totally new perspective on paper-writing and who would know this better than David? There are quite a few more colleagues out there who may not even realize what a huge boon they have been for me, such as Cathy Rankin, Richard Morris, Ralph Greenspan, Ed Kravitz or Eric Kandel.

Of course I owe a lot to all the colleagues in our lab. Bernd, Lisa and Tilman for making lunch-time so enjoyable, Doro and Uwe for stimulating discussions and sharing important knowledge. Gérard for his company inside and outside of the lab and for the best Canard I have ever tasted. Last but not least the scores of undergraduate and graduate students who helped pushing my flies when I was out of town.

Finally, I would not be writing this without the support of my parents and my partner, Diana, the most important people in and for my life.

9. Complete list of publications and abstracts

Peer-reviewed original research:

1. **Brembs, B.** * (2008): Mushroom-bodies regulate habit formation in *Drosophila*. J. Neurosci*. In prep.*
2. **Brembs, B.** * and Maye, A. (2008): Analyzing the temporal structure of spontaneous yaw torque behavior in *Drosophila.* JoVE *subm.*
3. **Brembs, B.*** and Plendl, W. (2008): Double dissociation of protein-kinase C and adenylyl cyclase manipulations on operant and classical learning in *Drosophila*. Curr. Biol. *re-subm.*
4. **Brembs, B.** * (2008): Operant learning of *Drosophila* at the torque meter. JoVE *16*. http://www.jove.com/index/Details.stp?ID=731, doi: 10.3791/731
5. **Brembs, B.**; Christiansen, F.; Pflüger, H.J. and Duch, C. (2007): Flight initiation and maintenance deficits in flies with genetically altered biogenic amine levels. J. Neurosci. *27*, 11122-11131
6. Maye, A.; Hsieh, C.; Sugihara, G. and **Brembs, B.*** (2007): Order in spontaneous behavior. PLoS One *2*: e443
7. **Brembs, B.*** and Hempel de Ibarra, N. (2006): Different parameters support discrimination and generalization in *Drosophila* at the flight simulator. Learn. Mem. *13*, 629-637
8. **Brembs B.*** and Wiener, J. (2006): Context generalization and occasion setting in *Drosophila* visual learning. Learn. Mem. *13*, 618-628
9. Phillips A.M.; Smart R.; Strauss R.; **Brembs B.** and Kelly, L.E. (2005): The *Drosophila black* enigma: the molecular and behavioural characterization of the *black*^{1} mutant allele. Gene *351C*, 131-142.
10. **Brembs B.***; Baxter D.A. and Byrne J.H. (2004): Extending *in vitro* conditioning in *Aplysia* to analyze operant and classical processes in the same preparation. Learn. Mem. *11*, 412-420.
11. **Brembs B.**; Lorenzetti F.D.; Reyes F.D.; Baxter D.A. and Byrne J.H. (2002): Operant Reward Learning in *Aplysia*: Neuronal Correlates and Mechanisms. Science *296*, 1706-1709.
12. Baier A.; Wittek B. and **Brembs B.*** (2002): *Drosophila* as a new model organism for the neurobiology of aggression? J. Exp. Biol. *205*, 1233-1240.
13. **Brembs B.*** and Heisenberg M. (2001): Conditioning with compound stimuli in *Drosophila* at the flight simulator. J. Exp. Biol. *204*, 2849-2859
14. **Brembs B.** and Heisenberg M. (2000): The Operant and the Classical in conditioned orientation of *Drosophila melanogaster* at the flight simulator. Learn. Mem. *7*, 104-115.

15. Cutts C.J.; **Brembs B.**; Metcalfe N.B. and Taylor A.C. (1999): Prior residence, territory quality and life-history strategies in juvenile Atlantic salmon (*Salmo salar* L.). J. Fish. Biol. *55*, 784-794.

Review articles:

Invited Reviews:

1. **Brembs B**.* (2008): The importance of being active. J. Neurogen. Spec. Issue. *subm.*
2. **Brembs B.*** (2003): Operant conditioning in invertebrates. Curr. Opin. Neurobiol. *13*, 710-717.
3. **Brembs B.*** (2003): Operant reward learning in *Aplysia*. Curr. Dir. Psychol. Sci. *12*, 218-221.

Reviews:

4. **Brembs B.*** (2008): Brains as output/input systems. *In prep.*
5. Heisenberg M.; Wolf R. and **Brembs B.** (2001): Flexibility in a single behavioral variable of *Drosophila*. Learn. Mem. *8*, 1-10
6. **Brembs B.*** (1996): Chaos, cheating and cooperation: potential solutions to the Prisoner's Dilemma. OIKOS *76*, 14-24.

Corresponding author

Book Chapters:

1. **Brembs B.** (2008): What can we learn from fruit fly learning? In: Guadagnoli M, de Belle, JS (eds) Biology, Brain & Movement, Elsevier Science, London, *in prep.*
2. **Brembs B.** (2008): Operant conditioning. In: Windhorst, U. Binder, M.D. and Hirokawa, N. (eds) Encyclopedia of Neuroscience. Springer, Berlin Heidelberg. *In press*.
3. Menzel R.; **Brembs, B.** and Giurfa M. (2006): Cognition in Invertebrates. In: Kaas, J.H. (ed.) Evolution of Nervous Systems. Chapter No. 1.26. Academic Press, Oxford; pp. 403-422
4. **Brembs B.** (2001): Hamilton's Theory. In: Brenner, S. and Miller, J. (eds) Encyclopedia of Genetics, Academic Press, London, New York; pp. 906-910.

Popular Science Articles:

1. **Brembs, B** (2008): Spontaneous actions and habitual responses in fruit flies. *The Naked Scientists*, BBC Radio, http://nakedscientists.com (*in press*)

Talks and Presentations:

Invited Presentations:

1. **11.06.2008:** The molecular basis and hierarchical organization of adaptive behavioral choice in *Drosophila*. BMC Neuroscience Seminars, Uppsala Biomedicinska Centrum, Uppsala University, Uppsala, Sweden
2. **03.-06.05.2008:** The molecular basis and hierarchical organization of predictive learning in *Drosophila*. CIN Selection Symposium, Werner Reichardt - Center für integrative Neurowissenschaften, Tübingen, Germany
3. **14.02.2008**: PLoS One/SciVee: Wissenschaftliche Video-Publikation. Medienforum "Videokommunikation in der Biotechnologie", IWF Wissen und Medien gGmbH, Göttingen, Germany
4. **08./09.01.2008:** Neurogenetic dissection of learning-by-doing in *Drosophila*. Symposium "Molecular Neurobiology of Behavior", Georg-August-Universität Göttingen, Germany
5. **27.11.007:** Dissecting learning-by-doing in *Drosophila*. Symposium in Systems Neuroscience, Ludwig-Maximilians Universität München, Germany.
6. **21.11.2007:** Genetic dissection of learning-by-doing in *Drosophila*. ALeRGiC Seminar Series, University of Sussex, Brighton, UK.
7. **23.10.2007:** Order in the spontaneous behavior of *Drosophila*. Interdisciplinary Seminar Series: "Irreversible Prozesse und Selbstorganisation", Institut für Physik, Humboldt Universität zu Berlin, Germany
8. **21.10.2007:** The generation effect in flies. Monthly Berlin meeting, Biologie-Netz.de, Berlin, Germany
9. **05.-09.06.2007:** *Aplysia* as an attractive alternative for analyzing agency? "Gastropod Neuroscience: Past Successes and Future Prospects." University of Washington, Friday Harbor Labs, San Juan Island, USA
10. **13.-15.03.2007:** Brains as Output/Input Systems. Janelia Farm Conference: "Insect Behavior: Small Brains, Big Functions", Janelia Farm Research Campus, USA
11. **15./16.01.2007**: Brains as Output/Input Systems. Symposium in Molecular Neurobiology, Friedrich Miescher Institute, Basel, Switzerland.
12. **17.-22.09.2006:** Brains as Output/Input Devices. XIII Summer School, Nicolás Cabrera Institute: "Biophysics of Biological Circuits: from Molecules to Networks." Universidad Autónoma de Madrid, Spain.
13. **19.-23.07.2004:** Discussant, Novartis Foundation Symposium No. 268 on "Molecular Mechanisms Influencing Aggressive Behaviours." London, England, UK.
14. **02.05.2002:** Operant reward learning in *Aplysia*, Lecture Series "Neurogenetics", Universität Würzburg, Germany

15. **Brembs, B.** (2008): Adenylyl cyclase and PKC differentiate operant and classical learning in *Drosophila*. Soc. Neurosci. Abstr.,

16. **Brembs, B.** (2008): Mushroom-bodies regulate habit formation in *Drosophila*. FENS Abstr. (talk)

17. **Brembs, B.** (2008): Neurogenetic dissection of learning-by-doing in *Drosophila*. Gordon Research Conference "Genes & Behavior", Barga, Italy

18. **Brembs, B.**; Christiansen, F.; Pflüger, H.J. and Duch, C. (2007): Flight motor performance deficits in flies with genetically altered biogenic amine levels. Soc. Neurosci. Abstr., 453.9 (talk)

19. **Brembs, B.** (2007): Mushroom-bodies regulate habit formation in *Drosophila*. 8th International Congress of Neuroethology, Vancouver, Canada

20. **Brembs, B.**; Maye, A; Hsieh, C. and Sugihara, G. (2007): Do fruit flies have free will? 8th International Congress of Neuroethology, Vancouver, Canada

21. **Brembs, B.** (2006): Operant and classical components interact hierarchically in *Drosophila* predictive learning. Soc. Neurosci. Abstr., 813.26

22. Christiansen, F.; Pflüger, J.; Duch, C.; and **Brembs, B.** (2006): Profound flight performance deficit in *Drosophila* lacking octopamine. FENS Abstr., vol.3, A218.2

23. **Brembs, B.**; Hsieh, C.; Sugihara, G. and Maye, A (2006): Do fruit flies have free will? FENS Abstr., vol.3, A233.7

24. Wiener, J.; Gerber, B.; Hempel de Ibarra, N.; Menzel, R. and **Brembs, B.** (2005): Occasion setting in *Drosophila* at the flight simulator. Soc. Neurosci. Abstr., 777.9

25. **Brembs, B.**; Maye, A. and Greggers, U. (2005): Order in spontaneous behavior. Soc. Neurosci. Abstr., 754.2.

26. Carbon, C.C.; Leder, H.; Weber, J.; Sander, T.; Trahms, L.; Grueter, M.; Grueter, T.; **Brembs, B.** and Lueschow, A. (2004): Specific impairments of configural processing in prosopagnosics. Soc. Neurosci. Abstr., 200.23.

27. **Brembs B.**; Baxter D.A. and Byrne J.H. (2004): Extending *in vitro* conditioning in *Aplysia* to analyze operant and classical processes in the same preparation. 7th International Congress of Neuroethology, Nyborg, Denmark.

28. Evans C.G; Jing J.; Proekt A., **Brembs B.**; Rosen S. and Cropper E.C. (2003): Frequency-dependent regulation of afferent transmission in the feeding circuitry of *Aplysia*. Soc. Neurosci. Abstr. 604.1.

29. **Brembs B.**; Wilkinson E.; Reyes F.; Baxter D.A. and Byrne J.H. (2001): Operant conditioning of feeding behavior in *Aplysia*. 6th International Congress of Neuroethology, Bonn, Germany.

30. **Brembs B.**; Wilkinson E.; Reyes F.; Baxter D.A. and Byrne J.H. (2001): Operant conditioning of feeding behavior in *Aplysia* using self-stimulation. Soc. Neurosci. Abstr. 644.19

31. Baxter D.A.; **Brembs B.** and Byrne J.H. (2001): Operant conditioning of feeding behavior in *Aplysia*. Cold Spring Harbor Symposium on Learning and Memory.

32. **Brembs B.**; Wilkinson E.; Reyes F.; Baxter D.A. and Byrne J.H. (2001): Operant conditioning using self-stimulation in *Aplysia*. In: Kreutzberg GW and Elsner N (eds) Göttingen Neurobiology Report 2001. Georg Thieme Verlag Stuttgart, New York

33. Baxter D.A.; Cai Y.; **Brembs B.** and Byrne J.H. (2000): Simulating physiological and morphological properties of neurons with SNNAP (Simulator for Neural Networks and Action Potentials). Soc. Neurosci. Abstr. 26:21.64.

34. **Brembs B.**; Wolf R. and Heisenberg M. (1999): Classical Questions in an Operant Learning Paradigm. In: Elsner N and Eysel U (eds) Göttingen Neurobiology Report 1999. Georg Thieme Verlag Stuttgart, New York: 545.

35. **Brembs B.**; Wolf R. and Heisenberg M. (1998): Operant and Classical Learning at the Flight Simulator: What is the Role of the Context? In: Elsner N and Wehner R (eds) New Neuroethology on the Move. Georg Thieme Verlag Stuttgart, New York: 514.

36. **Brembs B.**; Wolf R. and Heisenberg M. (1998): How different are operant and classical conditioning at the flight simulator? 5th International Congress of Neuroethology, San Diego, Ca.

37. Wolf R.; **Brembs B.**; Ernst R. and Heisenberg M. (1998)**:** Classification of learning in tethered flying *Drosophila*. In: Elsner N and Wehner R (eds) New Neuroethology on the Move. Georg Thieme Verlag Stuttgart, New York: 111 (talk)

38. **Brembs B.**; Wolf R.; Heisenberg M. (1997): Is operant behavior facilitating classical conditioning of *Drosophila* at the flight simulator? In: Elsner N, Waessle H (eds) Göttingen Neurobiology Report 1997. Georg Thieme Verlag Stuttgart, New York: 652.

10. Declaration of co-authors

The declarations listed below are copies or scans. I keep the original signatures.
Any publication not listed here either already contains the authors' contributions or is a single author publication.

Freie Universität Berlin

FB Biologie, Chemie, Pharmazie
Institut für Biologie
- Neurobiologie -

Freie Universität Berlin, Fachbereich Biologie, Chemie, Pharmazie
Institut für Biologie – Neurobiologie, Königin-Luise-Str. 28/30, D - 14195 Berlin

Dr. Björn Brembs

Königin-Luise-Str. **28/30**
14195 Berlin
Germany

Phone +49 30 838-54676
Fax +49 30 838-55455
E-Mail bjoern@brembs.net
Internet http://brembs.net

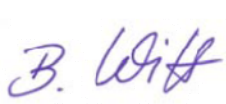
Berlin, July 2, 2008

Baier A.; Wittek B. and Brembs B. (2002)

Drosophila as a new model organism for the neurobiology of aggression?
J. Exp. Biol. 205, 1233-1240.

This paper describes the first neurobiological manipulations in fruit flies aimed to interfere with aggressive behaviors. All authors developed the experimental design and the method for behavioral scoring together. AB and BW performed the experiments and scored the behavior. BB evaluated the data. The manuscript was discussed by all authors and written by BB.

I agree with this statement on the contribution of the authors to the above publication.



A. Baier Dr. B. Wittek Dr. B. Brembs

Freie Universität Berlin

FB Biologie, Chemie, Pharmazie
Institut für Biologie
- Neurobiologie -

Freie Universität Berlin, Fachbereich Biologie, Chemie, Pharmazie
Institut für Biologie – Neurobiologie, Königin-Luise-Str. 28/30, D - 14195 Berlin

Dr. Björn Brembs

Königin-Luise-Str. 28/30
14195 Berlin
Germany

Phone +49 30 838-54676
Fax +49 30 838-55455
E-Mail bjoern@brembs.net
Internet http://brembs.net

Berlin, July 2, 2008

Brembs B.; Lorenzetti F.D.; Reyes F.D.; Baxter D.A. and Byrne J.H. (2002)

Operant Reward Learning in Aplysia: Neuronal Correlates and Mechanisms. Science 296, 1706-1709.

This paper describes a series of experiments from operant conditioning of feeding behavior in intact *Aplysia*, via physiology on the network level down to a single cell analogue of operant learning. The behavioural experiments were carried out by BB, the experiments on the network level were carried out by BB together with FDL and the single cell experiments were performed by FDL. FDR provided part of the behavioral data from control animals. The manuscript was discussed by all authors and written by BB.

I agree with this statement on the contribution of the authors to the above publication

Dr. B Brembs

Dr. FD Lorenzetti

FD Reyes

Dr. DA Baxter

Dr. JH Byrne

Freie Universität Berlin

FB Biologie, Chemie, Pharmazie
Institut für Biologie
- Neurobiologie -

Dr. Björn Brembs

Königin-Luise-Str. 28/30
14195 Berlin
Germany

Phone +49 30 838-54676
Fax +49 30 838-55455
E-Mail bjoern@brembs.net
Internet http://brembs.net

Berlin, July 2, 2008

Freie Universität Berlin, Fachbereich Biologie, Chemie, Pharmazie
Institut für Biologie – Neurobiologie, Königin-Luise-Str. 28/30, D - 14195 Berlin

Brembs B.*; Baxter D.A. and Byrne J.H. (2004)

Extending in vitro conditioning in Aplysia to analyze operant and classical processes in the same preparation. Learn. Mem. 11, 412-420.

This paper describes the development of a novel *in vitro* conditioning preparation of the *Aplysia* buccal ganglia, which includes both operant and classical components. All experiments were designed, carried out and analyzed by BB. The manuscript was discussed by all authors and written by BB.

I agree with this statement on the contribution of the authors to the above publication.

B. Brembs — Dr. B Brembs

Doug Baxter — Dr. DA Baxter

John H Byrne — Dr. JH Byrne

Freie Universität Berlin

FB Biologie, Chemie, Pharmazie
Institut für Biologie
- Neurobiologie -

Freie Universität Berlin, Fachbereich Biologie, Chemie, Pharmazie
Institut für Biologie – Neurobiologie, Königin-Luise-Str. 28/30, D - 14195 Berlin

Dr. Björn Brembs

Königin-Luise-Str. **28/30**
14195 Berlin
Germany

Phone +49 30 838-54676
Fax +49 30 838-55455
E-Mail bjoern@brembs.net
Internet http://brembs.net

Berlin, July 2, 2008

Phillips A.M.; Smart R.; Strauss R.; Brembs B. and Kelly, L.E. (2005)

The Drosophila black enigma: the molecular and behavioural characterization of the black1 mutant allele. Gene 351C, 131-142.

This paper contains the molecular genetic characterization of the *black* mutation in *Drosophila*, as well as a physiological and behavioral assessment of the visual defects in the mutant flies. The project was initiated by AMP. The behavioural experiments were carried out by BB and RSt. RSt evaluated the behavioural data. Sequencing of the mutant and generation of double mutants was the work of RSm. Enzyme assays and Western blotting were carried out by AMP. LEK performed the ERG experiments. Illustrations for the paper, and critical intellectual inputs relating to both the experimental procedures and manuscript preparation were provided by LEK. All non-behavioural experiments were performed in the LEK laboratory. The manuscript was discussed by all authors and written by AMP and LEK.

I agree with this statement on the contribution of the authors to the above publication.

A.M. Phillips R Smart R Strauss

Dr. AM Phillips Dr. R Smart Dr. R Strauss

B. Brembs Leonard E. Kelly.

Dr. B Brembs Dr. LE Kelly

Freie Universität Berlin

FB Biologie, Chemie, Pharmazie
Institut für Biologie
- Neurobiologie -

Freie Universität Berlin, Fachbereich Biologie, Chemie, Pharmazie
Institut für Biologie – Neurobiologie, Königin-Luise-Str. 28/30, D - 14195 Berlin

Dr. Björn Brembs

Königin-Luise-Str. 28/30
14195 Berlin
Germany

Phone +49 30 838-54676
Fax +49 30 838-55455
E-Mail bjoern@brembs.net
Internet http://brembs.net

Berlin, June 30, 2008

Brembs B. and Wiener, J. (2006)

Context generalization and occasion setting in Drosophila visual learning. Learn. Mem. 13, 618-628.

This paper describes experiments with wildtype and transgenic *Drosophila* which show that the classification of visual stimuli into context or occasion-setters depends on their relationship with reward or punishment. JW contributed data to Fig. 2. The remaining experiments were designed, carried out and analyzed by BB. The manuscript was discussed by all authors and written by BB.

I agree with this statement on the contribution of the authors to the above publication.

B. Brembs

Dr. B Brembs

J. Wiener

Dr. J Wiener

Freie Universität Berlin

FB Biologie, Chemie, Pharmazie
Institut für Biologie
- Neurobiologie -

Freie Universität Berlin, Fachbereich Biologie, Chemie, Pharmazie
Institut für Biologie – Neurobiologie, Königin-Luise-Str. 28/30, D - 14195 Berlin

Dr. Björn Brembs

Königin-Luise-Str. 28/30
14195 Berlin
Germany

Phone +49 30 838-54676
Fax +49 30 838-55455
E-Mail bjoern@brembs.net
Internet http://brembs.net

Berlin, June 30, 2008

Brembs, B. and Hempel de Ibarra, N. (2006)

Different parameters support discrimination and generalization in Drosophila at the flight simulator. Learn. Mem. 13, 629-637.

This paper describes experiments with wildtype *Drosophila* which show that flies use different parameters for the classification of visual stimuli into context or occasion-setters. All behavioral experiments were designed, performed and analyzed by BB. The parameter measurements of the color stimuli as well as the computation of the fly-subjective perception was performed by NH. The manuscript was discussed by all authors and written by BB.

I agree with this statement on the contribution of the authors to the above publication.

Dr. B Brembs

Dr. N Hempel de Ibarra

11. Publications submitted for the habilitation

The Journal of Experimental Biology 205, 1233–1240 (2002)
Printed in Great Britain © The Company of Biologists Limited
JEB4056

Drosophila as a new model organism for the neurobiology of aggression?

Andrea Baier*, Britta Wittek* and Björn Brembs†

Lehrstuhl für Genetik und Neurobiologie, Biozentrum, Am Hubland, D-97074 Würzburg, Germany

*These authors contributed equally to this work

†Author for correspondence at present address: Department of Neurobiology and Anatomy, W. M. Keck Center for the Neurobiology of Learning and Memory, University of Texas-Houston Medical School, Houston, Texas 77030, USA (e-mail: bjoern@brembs.net)

Accepted 22 February 2002

Summary

We report here the effects of several neurobiological determinants on aggressive behaviour in the fruitfly *Drosophila melanogaster*. This study combines behavioural, transgenic, genetic and pharmacological techniques that are well established in the fruitfly, in the novel context of the neurobiology of aggression. We find that octopamine, dopamine and a region in the *Drosophila* brain called the mushroom bodies, all profoundly influence the expression of aggressive behaviour. Serotonin had no effect. We conclude that *Drosophila*, with its advanced set of molecular tools and its behavioural richness, has the potential to develop into a new model organism for the study of the neurobiology of aggression.

Key words: *Drosophila melanogaster*, aggression, fighting behaviour, amine, mushroom body.

Introduction

Drosophila is the 'jack of all trades' in biology, but has not been studied in the context of the neurobiology of aggression. The fruitfly exhibits aggressive behaviour (Jacobs, 1960) and this behaviour is ethologically well characterized (Dow and von Schilcher, 1975; Jacobs, 1978; Lee and Hall, 2000; Skrzipek et al., 1979). The evolutionary relevance of this aggressive behaviour is also well established (Boake and Hoikkala, 1995; Boake and Konigsberg, 1998; Boake et al., 1998; Dow and von Schilcher, 1975; Hoffmann, 1988, 1989, 1994; Hoffmann and Cacoyianni, 1989; Ringo et al., 1983; Skrzipek et al., 1979; Zamudio et al., 1995). Finally, the ecological circumstances under which *Drosophila* exhibits territoriality and aggression have been examined in great detail (Hoffmann, 1987, 1988, 1989, 1994; Hoffmann and Cacoyianni, 1989, 1990). Under appropriate conditions, male flies try to occupy a food patch and defend it against other males, even in the laboratory. However, this aggressive behaviour in *Drosophila* has escaped the notice of most neurobiologists. Here we report the combination of ethological, ecological and evolutionary knowledge with molecular, genetic and pharmacological tools to manipulate the aggressive behaviour of *Drosophila melanogaster*.

To our knowledge, only two genetic factors have been reported to affect aggressive behaviour in *Drosophila*: the sex-determination hierarchy (SDH) and the β-alanine pathway. *fruitless* (*fru*) and *dissatisfaction* (*dsf*) mutants have been described as more aggressive than wild-type controls (Lee and Hall, 2000). Both genes are part of the SDH. Flies carrying mutant alleles of the *black* (*b*) gene appear less aggressive, whereas *ebony* (*e*) mutants appear more aggressive (Jacobs, 1978). The enzymes encoded by the two genes regulate β-alanine levels (*b* flies have reduced and *e* flies elevated levels).

It is straightforward to expect genes of the SDH to affect sex-specific behaviours, but the pathways by which they modulate that behaviour are largely unknown. One possibility could be *via* the regulation of small neuroactive molecules (such as β-alanine and the biogenic amines) and their receptors. Biogenic amines play a key role in the regulation of aggressive behaviour, not only in vertebrates, but also in arthropods (e.g. Edwards and Kravitz, 1997; Heinrich et al., 1999, 2000; Huber et al., 1997a,b; Kravitz, 2000; Schneider et al., 1996; Stevenson et al., 2000). The biogenic amine system in flies is well described (see Monastirioti, 1999). Most serotonin and dopamine mutants in *Drosophila* are either lethal or affect both serotonin and dopamine, due to their shared pathways of synthesis (e.g. Johnson and Hirsh, 1990; Lundell and Hirsh, 1994; Shen et al., 1993; Shen and Hirsh, 1994). However, established protocols are commonly used to manipulate the levels of these amines individually in the adult fly (Neckameyer, 1998; Vaysse et al., 1988). Octopamine null mutants have been generated and characterized (Monastirioti et al., 1996). Interestingly, certain octopamine and dopamine receptors are preferentially expressed in a prominent neuropil in the *Drosophila* brain called the mushroom bodies (Han et al., 1996, 1998). Thus, all of the prerequisites for a systematic analysis of the neurobiological factors involved in the expression of aggressive behaviour are available: (1) a considerable body of knowledge about the behaviour and its

ecological context, (2) circumstantial evidence about possible neurobiological factors involved in regulating the behaviour, and (3) methods for manipulating these factors and for quantifying the behaviour.

As a first attempt to characterize the effects of various possible neurobiological factors that might regulate aggression, we report here the results of a competition experiment. Six male flies competed for a food patch and three mated females. The experimental males were manipulated in one of various ways: by a classical mutation affecting β-alanine levels, a P-element mutation affecting octopamine levels, or insertion of transgenes affecting synaptic output from the mushroom bodies, or by pharmacological treatment affecting serotonin or dopamine levels, and then tested for their aggressive behaviour.

Materials and methods

Flies

Animals were kept on standard cornmeal/molasses medium (for recipe, see Guo et al., 1996) at 25 °C and 60 % humidity with a 16 h:8 h light:dark regime, except where noted. The females in all experiments were mated wild-type Canton S flies.

Mutants

Black^{1} and *ebony*^{1} mutant strains from the laboratory's 18 °C stock collection (provided by S. Benzer in 1970) were kept at 25 °C for at least two generations. The M18 P-element octopamine mutant and control stocks (Monastirioti et al., 1996) were kept at 25 °C for two generations after arrival.

Transgenes

Sweeney et al. (1995) developed a method that constitutively blocks synaptic transmission by expressing the catalytic subunit of bacterial tetanus toxin (Cnt-E) in target neurons in the *Drosophila* brain using the P[GAL4] technique (Brand and Perrimon, 1993). Inspired by the preferential expression of certain dopamine and octopamine receptors in the mushroom bodies (Han et al., 1996, 1998), we used the Cnt-E transgene to block synaptic output from the mushroom bodies (Sweeney et al., 1995). Expression of another transgene, an inactive form of the tetanus toxin light chain (imp-tntQ), controlled for deleterious effects of protein overexpression (Sweeney et al., 1995). The P[GAL4] line mb247 (Schulz et al., 1996) served as a mushroom body-specific GAL4 driver (Zars et al., 2000) for both toxins. The trans-heterozygote offspring from the GAL4 driver strain and the two UAS_{GAL4} reporter strains (Cnt-E and imp-tntQ) entered the study.

Pharmacological treatments

Drosophila from the wild-type strain Berlin (wtb) were treated as described by Neckameyer (1998) and Vaysse et al. (1988). Briefly, the animals were fed a sucrose solution containing either 10 mg ml^{-1} of the serotonin precursor 5HTP (5-hydroxy-tryptophan) or 10 mg ml^{-1} of the serotonin synthesis inhibitor pCPA (para-chlorophenylalanine) to manipulate serotonin levels. Effectiveness of the treatment was verified qualitatively with standard immunohistochemical techniques using rabbit serotonin antisera (data not shown; Buchner et al., 1986, 1988). Alternatively, the animals were treated with 1 mg ml^{-1} of the dopamine precursor L-DOPA (L-3,4-dihydroxyphenylalanine) or 10 mg ml^{-1} of the dopamine synthesis inhibitor 3IY (3-iodo-tyrosine) to manipulate dopamine levels. Effectiveness of the treatment was verified by observation of cuticle tanning. A dose of 10 mg ml^{-1} L-DOPA was lethal, confirming unpublished data from Wendy Neckameyer (St Louis University School of Medicine).

Experimental groups

Using the different stocks described above, we arranged six different groups of '*low*' *versus* '*high*' males, such that the respective amine or the amount of synaptic output from the mushroom bodies was manipulated to produce relative high- and low-level subgroups.

(1) *Wild-type Berlin (wtb)*

Wild-type Berlin flies are randomly assigned to a 'high' or a 'low' group. No difference between the subgroups is expected (**negative control**).

(2) *Serotonin (5ht)*

(a) Wild-type Berlin with 10 mg ml^{-1} 5HTP in sucrose solution. This treatment produces high levels of serotonin (5ht+).

(b) Wild-type Berlin with 10 mg ml–1 pCPA in sucrose solution. This treatment produces low levels of serotonin (5ht–).

(3) *Octopamine (oa)*

(a) M18 P-element parental stock, from which the jump-out below was generated (red eyed). This strain has normal levels of octopamine (Monastirioti et al., 1996) and will be denoted the '*high*' subgroup (oa+).

(b) M18 jump-out mutants. As tyramine-beta-hydroxylase (octopamine-producing enzyme) null mutants (white eyed), these flies have no detectable octopamine (Monastirioti et al., 1996) and will be denoted the '*low*' subgroup (oa–).

(4) *Dopamine (da)*

(a) Wild-type Berlin with 1 mg ml^{-1} L-DOPA in sucrose solution. This treatment produces high levels of dopamine (da+).

(b) Wild-type Berlin with 10 mg ml^{-1} 3-iodo-tyrosine in sucrose solution. This treatment produces low levels of dopamine (da–).

(5) *β-alanine (b/e)*

(a) *ebony* mutants with high β-alanine levels (*e*).

(b) *black* mutants with low β-alanine levels (*b*). This group serves as the **positive control**, as it is known that *e* flies are more aggressive than *b* flies (Jacobs, 1978).

Table 1. *Experimental time-course*

	Day										
	1	2	3	4	5	6	7	8	9	10	11
Put in vials	5ht	oa	wtb	mb	da	b/e					
	wtb	da	b/e	5ht	oa	mb					
Mark					5ht	oa	wtb	mb	da	b/e	
					wtb	da	b/e	5ht	oa	mb	
Record						5ht	oa	wtb	mb	da	b/e
						wtb	da	b/e	5ht	oa	mb

Two groups were treated in separate vials but in parallel each experimental day. Each group was treated in two replicates, starting with different flies on different days.

For abbreviations see Materials and methods.

(6) *Mushroom bodies (mb)*

(a) Offspring of P[GAL4] line mb247 with the UAS-IMP-tntQ line. This strain has normal levels of synaptic output from the mushroom bodies and will be referred to as the '*high*' subgroup (mb+).

(b) Offspring of P[GAL4] line mb247 with the UAS-Cnt-E line. This strain has no synaptic output from the mushroom bodies and will be called the '*low*' subgroup (mb–).

Thus, we arranged four experimental groups and two control groups. For each group, the two subgroups ('*high*' and '*low*') compete against each other in one recording chamber. Each group was tested twice with different animals.

Recording chambers

Aggression was studied in cylindrical cages similar to those used by Hoffmann (1987), i.e. 100 mm Petri dishes, top and bottom separated by a 40 mm high spacer (i.e. a cylindrical chamber of 100 mm diameter and 40 mm height). The bottom of the chamber was filled with 2 % agar to moisturize the chamber. Flies were introduced by gentle aspiration through a small hole in the spacer. A food patch (10 mm diameter, 12 mm high) was positioned in the centre of the chamber, containing a mixture of minced 2 % agar, apple juice, syrup and a live yeast suspension (after Reif, 1998), filled to the level of the rim of the containing vial. The chamber was placed in a Styrofoam box (used to ship biochemical reagents on dry ice; outer measurements: 275×275 mm, height, 250 mm; inner measurements: 215×215 mm, height, 125 mm) to standardize lighting conditions and to shield the chambers from movements by the experimenters. Two Styrofoam boxes with one chamber each were arranged underneath video cameras, focused on the food patch in a darkened room at 25 °C. Ring-shaped neon-lights (Osram L32W21C, power supply Philips BRC406) on top of the boxes provided homogenous illumination throughout the experiment.

Experimental time course

The stocks were treated completely in parallel (see Table 1). A 5 % sucrose solution (in *Drosophila* ringer) with or without added treatment was pipetted onto 5 pieces of filter paper snugly fitting in cylindrical (12×40 mm) vials before transferring newly eclosed (0–24 h) male flies into the vials. The flies were transferred into new vials with new solution and new filter paper on a daily basis for 5 days. Each group was treated in two replicates, starting with new flies on different days (see Table 1). On the fifth day, 4–6 flies per subgroup were briefly immobilised on a cold plate and marked with one small dot on the thorax in either green or white acrylic paint. At 08.00 h (1 h after lights-on) on the sixth day, the animals of the two groups treated in parallel were transferred into the recording chambers (three mated, but otherwise untreated, Canton S females, and six males, three from each paired subgroup) and placed underneath the video cameras under identical conditions to those used during the recording time, except that the video recorders (VCRs) were turned off. Continuing the parallel treatment of two groups per day, two video set-ups were used simultaneously ('left' and 'right'). After an acclimatisation period of 2 h, the VCRs were set to record. For each group, we recorded 4 h of fly behaviour, once in each location (yielding the two replicates for each group), resulting in 12 video tapes (see Table 2). Data from both replicates were pooled. Since each group was measured twice with six (3+3) experimental animals (males) for each recording, the total number of observed males was 6 animals×2 replicates×6 groups=72. Recording of the experiments was randomised across days.

Behavioural scoring

Only male–male interactions were counted. Mated females lose their receptivity to male advances and the males cease courting quickly, refraining from courting for a number of hours (courtship conditioning; e.g. Greenspan and Ferveur, 2000). Little courtship behaviour was thus observed after the acclimatisation period.

Behavioural scoring was done blind, before the colour codes on the flies' thoraces were decoded into '*high*' and '*low*'. An interaction between two males was classified as either aggressive or non-aggressive as defined by Hoffmann (1987). Briefly, we classified encounters that contained the previously described boxing, head-butting, lunging, wrestling, tussling, charging and chasing behaviours (Dow and von Schilcher,

Table 2. *Colour codes and recording dates*

Day	Number	Left	Number	Right
6	1	5ht+, green / 5ht–, white	2	wtb
7	3	oa+, green / oa–, white	4	da+, green / da–, white
8	5	wtb	6	e, green / b, white
9	7	mb–, green / mb+, white	8	5ht+, green / 5ht–, white
10	9	da+, green / da–, white	10	oa+, green / oa–, white
11	11	e, green / b, white	12	mb–, green / mb+, white

Each group was measured twice, once under each camera with different flies. Each of the 12 experiments was saved on individually numbered, 4 h video tapes. This table was used to break the code after the behavioural scoring had been done blindly.

For abbreviations see Materials and methods.

1975; Hoffmann, 1987, 1988, 1989, 1994; Hoffmann and Cacoyianni, 1989, 1990; Jacobs, 1978; Skrzipek et al., 1979) as aggressive. Encounters that only contained approach, leg contact, wing vibration or wing flapping were classified as non-aggressive. If the encounter was classified as aggressive, it was straightforward to discern the aggressor as one animal attacking and/or chasing the other. Non-aggressive encounters could usually not be classified directionally. Thus, with three '*high*' and three '*low*' animals in the recording chamber, any interaction between them falls into seven categories, listed below:

(1) High attacks, high aggressive encounter (1ag)
(2) High attacks, low aggressive encounter (2ag)
(3) High/high, non-aggressive encounter (3nonag)
(4) High/low, non-aggressive encounter (4nonag)
(5) Low/low, non-aggressive encounter (5nonag)
(6) Low attacks, high aggressive encounter (6ag)
(7) Low attacks, low aggressive encounter (7ag)

This design thus yielded seven values, one for each of the respective interaction categories, giving each of the six groups a characteristic aggression profile (Fig. 1A).

Data analysis

A log–linear analysis (delta=0.005, criterion for convergence=0.0005, maximum iterations 500) was performed over the 6×7 table of observed behavioural frequencies to determine the effect of the treatments on the distribution of behavioural classes. To normalize for the total number of encounters, two derived parameters were computed from the raw data. The first is the likelihood that an individual of one subgroup will attack during an encounter (attack probability, P_A). It is calculated as the fraction of all encounters in that group involving a '*high*' (or '*low*', respectively) animal, where such an animal was the aggressor:

$$P_A = \frac{\text{Number of '}subgroup\text{' attacking encounters}}{\text{Number of encounters with '}subgroup\text{' participation}}, \quad (1)$$

i.e.:

$$P_{A,high} = \frac{1ag + 2ag}{1ag + 2ag + 3nonag + 4nonag + 6ag} \quad (2)$$

and

$$P_{A,high} = \frac{6ag + 7ag}{2ag + 4nonag + 5nonag + 6ag + 7ag}. \quad (3)$$

Thus, P_A describes the probability that a given individual will act aggressively against any other individual it encounters. The second derived parameter assesses the representation of each subgroup in the total number of encounters (encounter probability, P_E). It is calculated analogously to the first parameter as the fraction of all encounters in a group, where an animal of a specific subgroup (i.e. '*high*' or '*low*') participated:

$$P_E = \frac{\text{Number of encounters with '}subgroup\text{' participation}}{\text{Total number of encounters in the group}}, \quad (4)$$

i.e.:

$$P_{E,high} = \frac{1ag + 2ag + 3nonag + 4nonag + 6ag}{1ag + 2ag + 3nonag + 4nonag + 5nonag + 6ag + 7ag} \quad (5)$$

and

$$P_{E,low} = \frac{2ag + 4nonag + 5nonag + 6ag + 7ag}{1ag + 2ag + 3nonag + 4nonag + 5nonag + 6ag + 7ag}. \quad (6)$$

Thus, P_E describes the probability that an individual of one subgroup will be a participant in an encounter.

While P_A can be said to describe the level of aggression of a certain subgroup, P_E can be perceived as a control measure for the overall number of interactions in that subgroup, as influenced by, for example, general activity, visual acuity, etc. After the data transformation, the resulting probabilities were tested against random distribution using χ^2 tests.

Results

We performed two 4 h experiments with four experimental and two control groups in each experiment. In all, 48 h of video tape were analysed containing 9881 encounters (an average of 3.4 encounters min^{-1} or 137.2 encounters $male^{-1}$). The two 4 h experiments were pooled for each group, yielding one 7-score aggression profile for each group (Fig. 1A). A log–linear analysis over the six groups and the seven behavioural classes yields a $P<0.0001$ (Pearson $\chi^2=6479.426$, d.f.=30), suggesting the various treatments were effective in changing the proportions of the different classes of encounters in each group.

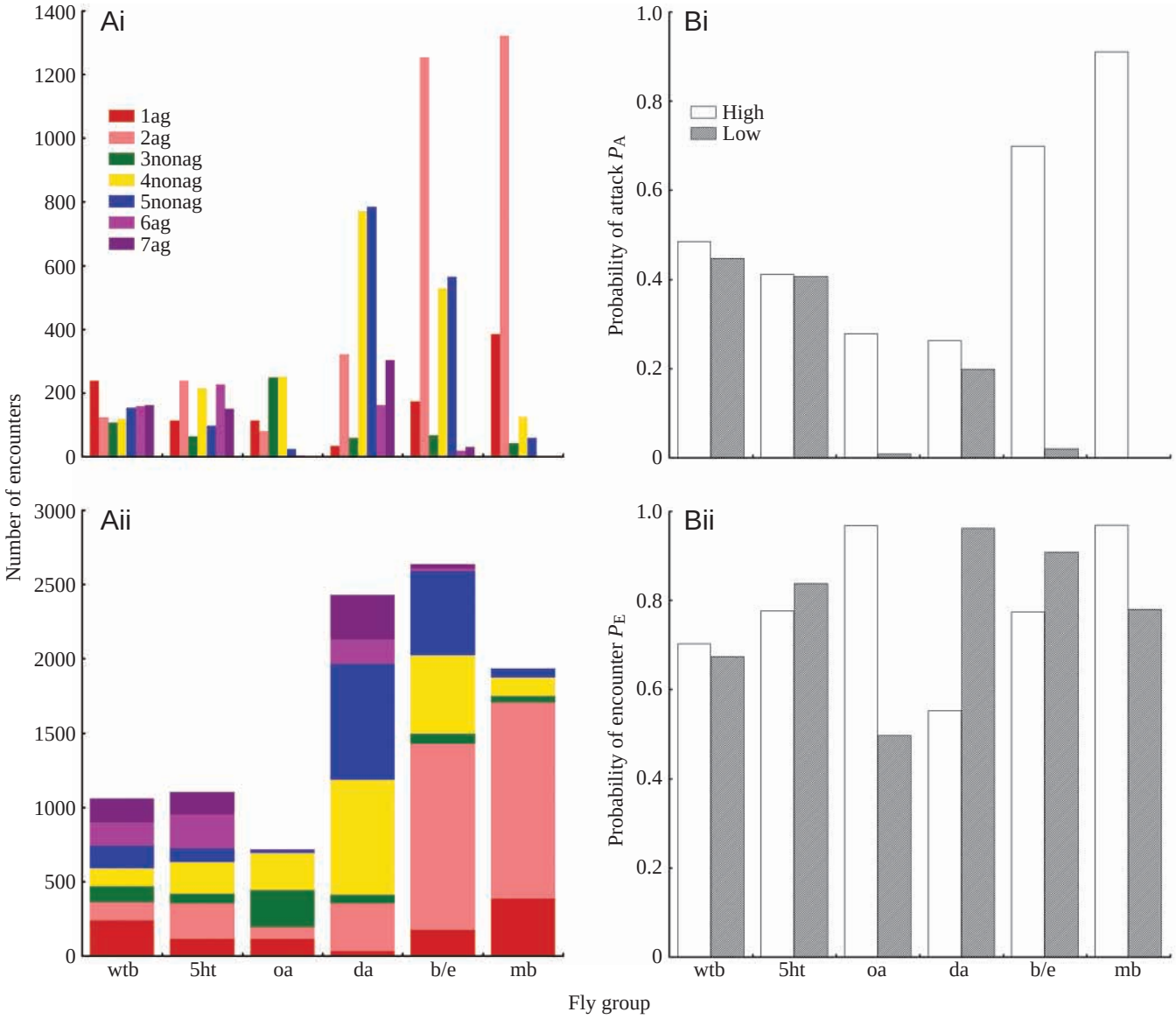


Fig. 1. Raw and derived data from all six groups. (A) Raw behavioural scores. Two different graphs depict the same data in order to facilitate the interpretation of the complex data structure obtained from our experiments. (Ai) Multiple bars graph, (Aii) single bar graph. *1ag*, high attacks, high aggressive encounter; *2ag*, high attacks, low aggressive encounter; *3nonag*, high/high attacks, non-aggressive encounter; *4nonag*, high/low attacks, non-aggressive encounter; *5nonag*, low/low attacks, non-aggressive encounter; *6ag*, low attacks, high aggressive encounter; *7ag*, low attacks, low aggressive encounter. (B) Derived probabilities. (Bi) The probability of attacking P_A. For each subgroup (*high*, *low*) the fraction of encounters where a member of that subgroup was the aggressor is calculated from the total number of subgroup encounters. (Bii) The probability of an encounter P_E. For each subgroup (*high*, *low*) the fraction of encounters (irrespective of classification) in which a member of that subgroup participated is calculated from the total number of encounters. Wtb, wild-type Berlin; 5ht, serotonin; oa, octopamine; da, dopamine; b/e, β-alanine; mb, mushroom bodies. See Materials and methods for details of behavioural classification and fly groups.

The raw data (Fig. 1A), reveal that the two control groups behaved according to our expectations. The wtb negative control shows a uniform distribution of aggressive encounters, whereas the β-alanine positive control is skewed towards the mutants with high levels of β-alanine (Fig. 1Ai).

The clearest effects among experimental groups were obtained from the octopamine mutants and the mb group. Both octopamine null mutants (oa–) and animals with inhibited mushroom bodies (mb–) are virtually non-aggressive (Fig. 1A). In Fig. 1Aii, the octopamine group seems similar to the wild-type control except for the missing values for 6ag and 7ag. However, while the oa+ animals appear to show a wild-type level of aggression, the mb+ animals show elevated levels of aggression compared to all other groups (Fig. 1A).

It also appears that our serotonin treatment had little effect on aggression (Fig. 1A).

The dopamine treatment appears to be somewhat effective in decreasing the number of aggressive encounters in animals with

Table 3. *χ^2-Statistics for derived probabilities*

	Probability of attack P_A		Probability of encounter P_E	
Fly group	χ^2 (Yates; d.f., 1)	*P* (Yates)	χ^2 (Yates; d.f., 1)	*P* (Yates)
Wild-type Berlin	0.01	=0.92	1.85	=0.17
Serotonin	0.31	=0.58	13.11	<0.0003
Octopamine	92.33	<0.0001	403.71	<0.0001
Dopamine	36.62	<0.0001	1109.17	<0.0001
β-alanine	2080.64	<0.0001	177.13	<0.0001
Mushroom bodies	3061.61	<0.0001	315.84	<0.0001

high levels of dopamine, while the animals with low levels of dopamine seem to have numbers of aggressive encounters similar to, if not slightly higher than, the wild-type controls. Obviously, the number of non-aggressive encounters in the dopamine-treated animals is strongly elevated (Fig. 1A). Interestingly, the two subgroups show inverted profiles for intra- and inter-subgroup aggression (i.e. 1ag/2ag and 6ag/7ag).

The total number of encounters also varies considerably between the different treatments (Fig. 1Aii).

With significant effects of our treatments on the distribution of the behaviours within each group, we can process the data in order to determine the effect of our treatments on the propensity of the animals to become aggressive. The fraction of all encounters involving a '*subgroup*' animal, where such an animal was the aggressor, is calculated (Fig. 1Bi; P_A, see Materials and methods). The P_A value allows us to estimate the effects of the treatments on aggression. χ^2 tests can be computed on P_A values to test the null hypothesis that our treatments had no effect on the probability of the fly being aggressive. Table 3 summarizes the χ^2 results for all six groups. The statistics confirm the effects already visible in the raw data (Fig. 1A): the two control groups (wtb and b/e) were consistent with our expectations. The obvious effect of octopamine null mutants being completely non-aggressive is corroborated by our statistical analysis, as are the extreme effects of expressing active and inactive tetanus toxin, respectively, in the flies' mushroom bodies (Fig. 1Bi). The serotonin treatment had no significant effect on the probability of the flies becoming aggressive during an encounter, despite the fact that we could verify the effectiveness of the treatment immunohistochemically (data not shown). The group in which the dopamine levels were manipulated shows a moderate, but statistically reliable, effect of high dopamine levels leading to a higher probability to attack in an encounter.

Despite the fact that most of our treatments have a record of influencing aggression in other animals, the possibility exists that the different treatments may have altered the number of aggressive encounters indirectly by altering the total number of encounters, through other factors such as general activity, visual acuity, etc. The distribution of encounters over the subgroups, P_E, should reveal such candidate variables. For instance, if the treatment rendered the animals of one subgroup inactive, the P_E of that subgroup should be smaller than the P_E of the other subgroup. If the obtained aggression scores were but a reflection of asymmetric P_E values, they should follow the pattern of P_E asymmetry. Fig. 1Bii depicts the distribution of encounters over the two subgroups, independently of encounter classification. Again, χ^2 statistics were performed and summarized in Table 2. All treatments led to a significant asymmetry in P_E between subgroups, with the exception of the negative wtb controls. However, the pattern of asymmetry does not seem to match the pattern of asymmetry in the level of aggression (see Discussion).

Discussion

Most importantly for this first study of the effects of various treatments on *Drosophila* aggression, the animals in the control groups behaved exactly as expected: no differences were detected among the subgroups of the wtb negative control, and previously published higher aggression levels in the *ebony* (high β-alanine) than in the *black* (low β-alanine) flies (Jacobs, 1978) could be reproduced. These findings corroborate our pilot studies in which we repeatedly observed the same pattern (A. Baier, B. Wittek and B. Brembs, unpublished data).

Octopamine null mutants exhibit strongly reduced aggression, as do flies with low levels of synaptic output from their mushroom bodies. Interestingly, certain types of octopamine and dopamine receptors are preferentially expressed in the mushroom bodies of wild-type flies (Han et al., 1996, 1998). It is tempting to interpret this phenocopy of the octopamine mutants as resulting from Kenyon cells being the major regulators of octopamine- (and/or dopamine-) mediated aggression. Recently, temperature sensitive shibire^{ts1} constructs have been developed to conditionally block synaptic transmission (e.g. Dubnau et al., 2001; Kitamoto, 2001; McGuire et al., 2001; Waddell et al., 2000). Unfortunately, at the time of our experiments, the shibire^{ts1} constructs were not yet available. Future experiments definitely should include shibire^{ts1} constructs in order to replicate our mb– results, examine the high levels of aggression in the mb+ flies and look for other brain areas involved in aggression. Replication of our results using the shibire^{ts1} constructs would also eliminate the possible explanation that the expression of tetanus toxin anywhere in the fly's brain abolishes aggressive behaviour and solve the problem of UAS promoter leakiness. The octopamine result is conspicuous in another respect: it is consistent with studies in crickets, where depletion of octopamine and dopamine decreases aggressiveness (Stevenson et al., 2000), but contrasts with studies in crustaceans, where high octopamine levels tend to bias behaviour towards submissiveness (Antonsen and Paul, 1997; Heinrich et al., 2000; Huber et al., 1997a).

The high aggression observed in the mb+ animals is difficult to interpret. In principle, the inactive toxin should not have any effect on the secretion of neurotransmitter at the synapse. More likely is an insertion effect of the P-element containing the imp-tntQ transgene. In that case it would be extremely interesting to characterize the genetic environment within which the P-element lies in order to find the gene responsible for such aggressiveness. One may argue that high aggressiveness by flies of one subgroup may produce low aggression in the respective

other subgroup. In the case of the mb group, this is unlikely, because there still should be at least some aggression between mb– animals, even if mb+ animals attacked every other male they encountered. Moreover, mb– animals seemed unaffected by the repeated attacks from mb+ males and kept coming back to the patch soon after an mb+ male chased it off (the reason for the high 2ag value in Fig. 1). However, mb– animals were never observed to be the aggressor. It thus seems more likely that the high frequency of attacks by mb+ males is due to a combination of high levels of aggression due to insertion effects of the imp-tntQ transgene and returning mb– males repeatedly eliciting aggressive behaviours in the mb+ males.

Our serotonin treatment has no significant effect on aggression, despite the fact that we could verify the effectiveness of the treatment immunohistochemically (data not shown). Also, Vaysse et al. (1988) observed effects on learning and memory after identical treatment, indicating that this pharmacological manipulation of serotonin levels in principle can have behavioural effects. Moreover, we observed a noticeable increase in activity in the 5ht– flies, a subjective impression that is corroborated by the significantly increased P_E of this subgroup (Fig. 1Bii). Nevertheless, the possibility remains that the observed difference in serotonin immunoreactivity was not high enough to generate significant differences in aggression, although it was high enough to affect other behaviours. The lack of serotonergic effect on aggression was also repeatedly observed in our pilot studies (A. Baier, B. Wittek and B. Brembs, unpublished data). Lee and Hall (2001) have reported that the pattern of serotonergic cells in the *Drosophila* brain is unaltered in the more aggressive *fru* mutants, confirming the idea that serotonin is not crucial for regulation of aggressive behaviour in the fly. The serotonin results presented here are also consistent with data in crickets, where serotonin depletion appears to have no effect (Stevenson et al., 2000); they contrast with data in crustaceans, where injections of serotonin increase the level of aggressive behaviour (Edwards and Kravitz, 1997; Huber et al., 1997a,b; Kravitz, 2000). Our serotonin data thus parallel our octopamine data in conforming with insect data but contrasting with observations in crustaceans. Perhaps aminergic control of aggression functions fundamentally differently in those two arthropod groups?

Our dopamine treatment had complex effects. The absolute number of non-aggressive encounters appears elevated compared to the wild-type controls (Fig. 1A), reducing overall aggression probabilities (Fig. 1Bi; P_A). Also, while the raw data indicate higher aggression scores in the animals with low dopamine (Fig. 1Ai), the P_A is higher in animals with high dopamine levels (Fig. 1Bi). Taking the number of encounters that each subgroup experiences (Fig. 1Bii, P_E) into account, it seems as if the higher raw scores for the '*low*' dopamine animals is generated by the higher P_E in this subgroup. Once that factor is accounted for (Fig. 1Bi), the perceived difference between raw and derived data disappears.

A general point of concern is possible side effects of our treatments. Both *e* and *b* flies exhibit varying degrees of visual impairment (A. Baier, B. Wittek and B. Brembs, unpublished data; Heisenberg, 1971, 1972; Hovemann et al., 1998; Jacobs, 1978), with *e* flies showing more severe defects than *b* flies (A. Baier, B. Wittek and B. Brembs, unpublished data; Jacobs, 1978). Without screening pigments (i.e. *white*$^-$), the M18 octopamine jump-out mutants are expected to have severely impaired vision compared with the control strain still carrying the P-element. Also, the extent by which the treatments may affect general activity is largely unknown (but see Martin et al., 1998). One may assume that a subgroup's P_E should reflect overall activity. Not surprisingly, the more visually impaired *e* and oa– flies have lower P_E values than the *b* and oa+ subgroups, respectively (Fig. 1Bii). However, the probability to attack seems entirely unaffected by this measure of general activity, as the relationships are reversed. Moreover, both the dopamine and the mushroom body groups show a higher probability to attack in the respective '*high*' subgroup (Fig. 1Bi), but their P_E values are inverted with respect to their P_A values (Fig. 1Bii). Thus, while both vision and general activity may influence aggression, those factors seem to have only marginal effects compared to the determinants studied here.

Of course, this study is only a beginning. We did not examine encounter duration, behavioural composition or opponent identity/recognition, let alone investigate potential mechanisms as to how the identified factors might exert their effects. However, our method successfully reproduced published data (the e/b group) and yielded new insights into the neurobiological determinants of aggression in *Drosophila melanogaster*. Serotonin appears to have no effect, while dopamine, octopamine and the mushroom bodies could be linked to the promotion of aggressive behaviour. We hope that our work will inspire others to exploit *Drosophila's* numerous technical advantages for studying the neurobiology of aggression.

We are very grateful to Martin Heisenberg for providing laboratory space, flies, equipment, intellectual support and comments on the manuscript, to Dieter Dudacek for immunohistochemistry, to Troy Zars for providing the P[GAL4] driver and UAS toxin lines, to Maria Monastirioti for providing the M18 stocks, to Wendy Neckameyer for most helpful discussions on the use of dopamine pharmacology, to Jay Hirsh for advice on serotonin and dopamine mutants, to Marcus Reif for helpful support on food-patch medium, to Reinhard Wolf for supplying 'Apfelmost' for the food-patch medium, to David Pettigrew, Randall Hayes, Gregg Phares, Gabi Putz, Robin Hiesinger, Sean McGuire and Douglas Armstrong for discussion and comments on the manuscript and to Hans Kaderaschabek and his team for designing and producing our special equipment. We are specially grateful to Edward Kravitz. This study would probably never have been initiated if it had not been for his curiosity and enthusiastic support. We owe much to his constant feedback and his participation in our very constructive discussions.

References

Antonsen, B. L. and Paul, D. H. (1997). Serotonin and octopamine elicit

stereotypical agonistic behaviors in the squat lobster *Munida quadrispina* (Anomura, Galatheidae). *J. Comp. Physiol. A* **181**, 501–510.
Boake, C. R. B. and Hoikkala, A. (1995). Courtship Behavior and Mating Success of Wild-Caught *Drosophila-Silvestris* Males. *Anim. Behav.* **49**, 1303–1313.
Boake, C. R. B. and Konigsberg, L. (1998). Inheritance of male courtship behavior, aggressive success, and body size in *Drosophila silvestris*. *Evolution* **52**, 1487–1492.
Boake, C. R. B., Price, D. K. and Andreadis, D. K. (1998). Inheritance of behavioural differences between two interfertile, sympatric species, *Drosophila silvestris* and *D. heteroneura*. *Heredity* **80**, 642–650.
Brand, A. H. and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118**, 401–415.
Buchner, E., Bader, R., Buchner, S., Cox, J., Emson, P. C., Flory, E., Heizmann, C. W., Hemm, S., Hofbauer, A. and Oertel, W. H. (1988). Cell-specific immuno-probes for the brain of normal and mutant *Drosophila melanogaster*. I. Wild type visual system. *Cell Tissue Res.* **253**, 357–370.
Buchner, E., Buchner, S., Crawford, G., Mason, W. T., Salvaterra, P. M. and Sattelle, D. B. (1986). Choline Acetyltransferase-Like Immunoreactivity in the Brain of *Drosophila melanogaster*. *Cell Tissue Res.* **246**, 57–62.
Dow, M. A. and von Schilcher, F. (1975). Aggression and mating success in *Drosophila melanogaster*. *Nature* **254**, 511–512.
Dubnau, J., Grady, L., Kitamoto, T. and Tully, T. (2001). Disruption of neurotransmission in *Drosophila* mushroom body blocks retrieval but not acquisition of memory. *Nature* **411**, 476–480.
Edwards, D. H. and Kravitz, E. A. (1997). Serotonin, social status and aggression. *Curr. Opin. Neurobiol.* **7**, 812–819.
Greenspan, R. J. and Ferveur, J. F. (2000). Courtship in Drosophila. *Annu. Rev. Genet.* **34**, 205–232.
Guo, A., Liu, L., Xia, S.-Z., Feng, C.-H., Wolf, R. and Heisenberg, M. (1996). Conditioned visual flight orientation in *Drosophila*; Dependence on age, practice and diet. *Learn. Mem.* **3**, 49–59.
Han, K. A., Millar, N. S. and Davis, R. L. (1998). A novel octopamine receptor with preferential expression in *Drosophila* mushroom bodies. *J. Neurosci.* **18**, 3650–3658.
Han, K. A., Millar, N. S., Grotewiel, M. S. and Davis, R. L. (1996). DAMB, a novel dopamine receptor expressed specifically in *Drosophila* mushroom bodies. *Neuron* **16**, 1127–1135.
Heinrich, R., Braunig, P., Walter, I., Schneider, H. and Kravitz, E. A. (2000). Aminergic neuron systems of lobsters: morphology and electrophysiology of octopamine-containing neurosecretory cells. *J. Comp. Physiol. A* **186**, 617–629.
Heinrich, R., Cromarty, S. I., Horner, M., Edwards, D. H. and Kravitz, E. A. (1999). Autoinhibition of serotonin cells: an intrinsic regulatory mechanism sensitive to the pattern of usage of the cells. *Proc. Natl. Acad. Sci. USA* **96**, 2473–2478.
Heisenberg, M. (1971). Separation of receptor and lamina potentials in the electroretinogram of normal and mutant *Drosophila*. *J. Exp. Biol.* **55**, 85–100.
Heisenberg, M. (1972). Comparative behavioral studies on two visual mutants of *Drosophila*. *J. Comp. Physiol.* **80**, 119–136.
Hoffmann, A. A. (1987). A Laboratory Study of Male Territoriality in the Sibling Species *Drosophila melanogaster* and *Drosophila simulans*. *Anim. Behav.* **35**, 807–818.
Hoffmann, A. A. (1988). Heritable Variation for Territorial Success in 2 *Drosophila melanogaster* Populations. *Anim. Behav.* **36**, 1180–1189.
Hoffmann, A. A. (1989). Geographic variation in the territorial success of *Drosophila melanogaster* males. *Behav Genet* **19**, 241–255.
Hoffmann, A. A. (1994). Genetic Analysis of territoriality in *Drosophila melanogaster*. In *Quantitative Genetic Studies of Behavioural Evolution* (ed. C. R. B. Boake), pp. 188–205. Chicago: Chicago University Press.
Hoffmann, A. A. and Cacoyianni, Z. (1989). Selection for Territoriality in *Drosophila melanogaster* – Correlated Responses in Mating Success and Other Fitness Components. *Anim. Behav.* **38**, 23–34.
Hoffmann, A. A. and Cacoyianni, Z. (1990). Territoriality in *Drosophila melanogaster* as a Conditional Strategy. *Anim. Behav.* **40**, 526–537.
Hovemann, B. T., Ryseck, R. P., Walldorf, U., Stortkuhl, K. F., Dietzel, I. D. and Dessen, E. (1998). The *Drosophila* ebony gene is closely related to microbial peptide synthetases and shows specific cuticle and nervous system expression. *Gene* **221**, 1–9.
Huber, R., Orzeszyna, M., Pokorny, N. and Kravitz, E. A. (1997a). Biogenic amines and aggression: experimental approaches in crustaceans. *Brain Behav. Evol.* **50, Suppl. 1**, 60–68.
Huber, R., Smith, K., Delago, A., Isaksson, K. and Kravitz, E. A. (1997b). Serotonin and aggressive motivation in crustaceans: altering the decision to retreat. *Proc. Natl. Acad. Sci. USA* **94**, 5939–5942.
Jacobs, M. E. (1960). Influence of light on mating of *Drosophila melanogaster*. *Ecology* **41**, 182–188.
Jacobs, M. E. (1978). Influence of beta-alanine on mating and territorialism in *Drosophila melanogaster*. *Behav. Genet.* **8**, 487–502.
Johnson, W. A. and Hirsh, J. (1990). Binding of a *Drosophila* POU-domain protein to a sequence element regulating gene expression in specific dopaminergic neurons. *Nature* **343**, 467–470.
Kitamoto, T. (2001). Conditional modification of behavior in *Drosophila* by targeted expression of a temperature-sensitive shibire allele in defined neurons. *J. Neurobiol.* **47**, 81–92.
Kravitz, E. A. (2000). Serotonin and aggression: insights gained from a lobster model system and speculations on the role of amine neurons in a complex behavior. *J. Comp. Physiol. A* **186**, 221–238.
Lee, G. and Hall, J. C. (2000). A newly uncovered phenotype associated with the fruitless gene of *Drosophila melanogaster*: aggression-like head interactions between mutant males. *Behav. Genet.* **30**, 263–275.
Lee, G. and Hall, J. C. (2001). Abnormalities of male-specific FRU protein and serotonin expression in the CNS of *fruitless* mutants in *Drosophila*. *J. Neurosci.* **21**, 513–526.
Lundell, M. J. and Hirsh, J. (1994). Temporal and spatial development of serotonin and dopamine neurons in the *Drosophila* CNS. *Dev. Biol.* **165**, 385–396.
Martin, J. R., Ernst, R. and Heisenberg, M. (1998). Mushroom bodies suppress locomotor activity in *Drosophila melanogaster*. *Learn. Mem.* **5**, 179–191.
McGuire, S. E., Le, P. T. and Davis, R. L. (2001). The role of *Drosophila* mushroom body signaling in olfactory memory. *Science* **293**, 1330–1333.
Monastirioti, M. (1999). Biogenic amine systems in the fruit fly *Drosophila melanogaster*. *Microsc. Res. Tech.* **45**, 106–121.
Monastirioti, M., Linn, C. E., Jr and White, K. (1996). Characterization of *Drosophila* tyramine beta-hydroxylase gene and isolation of mutant flies lacking octopamine. *J. Neurosci.* **16**, 3900–3911.
Neckameyer, W. S. (1998). Dopamine modulates female sexual receptivity in *Drosophila melanogaster*. *J. Neurogenet.* **12**, 101–114.
Reif, M. (1998). *The Evolution of Learning*. PhD thesis, University of Würzburg, pp. 131.
Ringo, J., Kananen, M. K. and Wood, D. (1983). Aggression and Mating Success in 3 Species of *Drosophila*. *Z. Tierpsych. – J. Comp. Ethol.* **61**, 341–350.
Schneider, H., Budhiraja, P., Walter, I., Beltz, B. S., Peckol, E. and Kravitz, E. A. (1996). Developmental expression of the octopamine phenotype in lobsters, *Homarus americanus*. *J. Comp. Neurol.* **371**, 3–14.
Schulz, R. A., Chromey, C., Lu, M. F., Zhao, B. and Olson, E. N. (1996). Expression of the D-MEF2 transcription in the *Drosophila* brain suggests a role in neuronal cell differentiation. *Oncogene* **12**, 1827–1831.
Shen, J., Beall, C. J. and Hirsh, J. (1993). Tissue-specific alternative splicing of the *Drosophila* dopa decarboxylase gene is affected by heat shock. *Mol. Cell Biol.* **13**, 4549–4555.
Shen, J. and Hirsh, J. (1994). Cis-regulatory sequences responsible for alternative splicing of the *Drosophila* dopa decarboxylase gene. *Mol. Cell Biol.* **14**, 7385–7393.
Skrzipek, K. H., Kroner, B. and Hager, H. (1979). Inter-Male Agression in *Drosophila melanogaster* – Laboratory Study. *Z. Tierpsych. – J. Comp. Ethol.* **49**, 87–103.
Stevenson, P. A., Hofmann, H. A., Schoch, K. and Schildberger, K. (2000). The fight and flight responses of crickets depleted of biogenic amines. *J. Neurobiol.* **43**, 107–120.
Sweeney, S. T., Broadie, K., Keane, J., Niemann, H. and O'Kane, C. J. (1995). Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron* **14**, 341–351.
Vaysse, G., Galissie, M. and Corbiere, M. (1988). Induced variation of serotonin in *Drosophila melanogaster* and its relation to learning performance. *J. Comp. Psychol.* **102**, 225–229.
Waddell, S., Armstrong, J. D., Kitamoto, T., Kaiser, K. and Quinn, W. G. (2000). The amnesiac gene product is expressed in two neurons in the *Drosophila* brain that are critical for memory. *Cell* **103**, 805–813.
Zamudio, K. R., Huey, R. B. and Crill, W. D. (1995). Bigger isn't always better – body-size, developmental and parental temperature and male territorial success in *Drosophila melanogaster*. *Anim. Behav.* **49**, 671–677.
Zars, T., Fischer, M., Schulz, R. and Heisenberg, M. (2000). Localization of a short-term memory in Drosophila. *Science* **288**, 672–675.

mediate the biological activities of other natural products with metabolic effects. For example, the activation of hPXR-SXR by the hyperforin present in the herbal antidepressant St. John's Wort results in undesirable effects on drug metabolism (*30*, *31*). It is an intriguing possibility that further characterization of the effects of natural products on such receptors will identify additional agents that, like guggulsterone, have more desirable activities.

References and Notes

1. D. J. Parks *et al.*, *Science* **284**, 1365 (1999).
2. M. Makishima *et al.*, *Science* **284**, 1362 (1999).
3. H. Wang, J. Chen, K. Hollister, L. C. Sowers, B. M. Forman, *Mol. Cell* **3**, 543 (1999).
4. C. J. Sinal *et al.*, *Cell* **102**, 731 (2000).
5. B. M. Forman *et al.*, *Cell* **81**, 687 (1995).
6. A. M. Zavacki *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 7909 (1997).
7. W. R. Howard, J. A. Pospisil, E. Njolito, D. J. Noonan, *Toxicol. Appl. Pharmacol.* **163**, 195 (2000).
8. P. R. Maloney *et al.*, *J. Med. Chem.* **43**, 2971 (2000).
9. G. V. Satyavati, *Indian J. Med. Res.* **87**, 327 (1988).
10. S. Dev, *Environ. Health Perspect.* **107**, 783 (1999).
11. S. Nityanand, J. S. Srivastava, O. P. Asthana, *J. Assoc. Physicians India* **37**, 323 (1989).
12. R. B. Singh, M. A. Niaz, S. Ghosh, *Cardiovasc. Drugs Ther.* **8**, 659 (1994).
13. S. Chander, A. K. Khanna, N. K. Kapoor, *Phytotherapy Res.* **10**, 508 (1996).
14. N. L. Urizar, D. H. Dowhan, D. D. Moore, *J. Biol. Chem.* **275**, 39313 (2000).
15. T. T. Lu *et al.*, *Mol. Cell* **6**, 507 (2000).
16. B. Goodwin *et al.*, *Mol. Cell* **6**, 517 (2000).
17. J. Grober *et al.*, *J. Biol. Chem.* **274**, 29749 (1999).
18. J. L. Staudinger *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 3369 (2001).
19. W. Xie *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **98**, 3375 (2001).
20. J. Staudinger, Y. Liu, A. Madan, S. Habeebu, C. D. Klaassen, *Drug Metab. Dispos.* **29**, 1467 (2001).
21. D. P. Wang *et al.*, *J. Lipid Res.* **37**, 1831 (1996).
22. M. Crestani, A. Sadeghpour, D. Stroup, G. Galli, J. Y. Chiang, *Biochem. Biophys. Res. Commun.* **225**, 585 (1996).
23. S. K. Cheema, L. B. Agellon, *J. Biol. Chem.* **275**, 12530 (2000).
24. K. von Bergmann, J. Fierer, H. Y. Mok, S. M. Grundy, *Antimicrob. Agents Chemother.* **19**, 342 (1981).
25. E. E. Ohnhaus, B. Kirchhof, E. Peheim, *Clin. Pharmacol. Ther.* **25**, 591 (1979).
26. J. Feely, M. Clee, L. Pereira, E. Guy, *Br. J. Clin. Pharmacol.* **16**, 195 (1983).
27. L. Bachs, A. Pares, M. Elena, C. Piera, J. Rodes, *Gastroenterology* **102**, 2077 (1992).
28. D. E. Cummings *et al.*, *Nature* **382**, 622 (1996).
29. J. J. Repa *et al.*, *J. Biol. Chem.* **275**, 39685 (2000).
30. L. B. Moore *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **97**, 7500 (2000).
31. J. M. Wentworth, M. Agostini, J. Love, J. W. Schwabe, V. K. Chatterjee, *J. Endocrinol.* **166**, R11 (2000).
32. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
33. Guggulsterones [GS, *cis*- and *trans*-4,17(20)-pregnadiene-3,16-dione] were obtained from Steraloids (Newport, RI) and dissolved in dimethyl sulfoxide (DMSO).
34. Y. K. Lee, K. L. Parker, H. S. Choi, D. D. Moore, *J. Biol. Chem.* **274**, 20869 (1999).
35. G. M. Williams, M. F. Laspia, V. C. Dunkel, *Mutat. Res.* **97**, 359 (1982).
36. B. L. Kreamer *et al.*, *In Vitro Cell Dev. Biol.* **22**, 201 (1986).
37. R. Enat *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **81**, 1411 (1984).
38. For real-time quantitative PCR, reaction mixes included a 200 nM final concentration of a SHP-specific TaqMan probe (5′ ATGTGCCAGGCCTCCGTGCCT) labeled with 6-carboxy fluorescein (FAM) reporter fluorescent dye, and a 50 nM and 300 nM final concentration of forward (5′ GTACCTGAAGGGCACGATCC) and reverse (5′ AGCCTCCTGTTGCAGGTGT) primers, respectively. For analysis, 1 ng of total RNA isolated from primary hepatocytes was used per reaction. The cycle parameters included a reverse transcription step at 48°C for 30 min, followed by 40 cycles of 95°C denaturation and 60°C annealing and extension. The 18S rRNA was used for the endogenous control.
39. For FRET analysis, the human FXR ligand-binding domain (LBD) (amino acids 244 to 472) was expressed as a GST-FXR-LBD fusion protein (glutathione S-transferase fused to FXR-LBD) in DH5α and purified using glutathione beads. The FRET assay was performed by incubating 8 nM of GST-FXR-LBD, 8 nM of Europium-labeled antibody to GST (Wallac, PerkinElmer Life Sciences, Boston, MA), 16 nM biotin-SRC-1 peptide [5′-biotin-CPSSHSSLTERHKILHRLLQEGSPS-CONH2] (*32*), 20 nM allophycocyanin conjugated streptavidin (APC-SA) (Wallac) in FRET assay buffer (20 mM KH_2PO_4/K_2HPO_4 (pH 7.3), 150 mM NaCl, 2 mM CHAPS detergent, 2 mM EDTA, 1 mM dithiothreitol (DTT) in the presence of the test compound(s) for 2 to 4 hours at room temperature. Data were collected using an LJL Analyst (Molecular Devices, Sunnyvale, CA). The results are expressed as 1000*(665 nm/615 nm).
40. Experimental diets consisted of control diet (TEKLAD 7001, Harlan Teklad, Madison, WI) supplemented with 2% cholesterol. Male 8- to 12-week-old mice were used for all experiments and were allowed water ad libitum. Z-Guggulsterone was resuspended in 0.2-ml saline and administered to mice by oral gavage. Control animals received the same amount of saline. At the end of the experiment, mice fasted for 4 hours, after which time livers were harvested and snap-frozen in liquid nitrogen and then stored at −80°C until use.
41. S. E. Carlson, S. Goldfarb, *Clin. Chim. Acta* **79**, 575 (1977).
42. M. Yokode, R. E. Hammer, S. Ishibashi, M. S. Brown, J. L. Goldstein, *Science* **250**, 1273 (1990).
43. J. Folch, M. Lees, G. H. Sloane Stanley, *J. Biol. Chem.* **226**, 497 (1957).
44. C. C. Allain, L. S. Poon, C. S. Chan, W. Richmond, P. C. Fu, *Clin. Chem.* **20**, 470 (1974).

45. We thank B. Wagner, J. Repa, and J. T. Lin for information and helpful suggestions. Supported by grants from the National Institute of Diabetes and Digestive and Kidney Diseases and USDA (to D.D.M.), an NIGMS (National Institute of General Medical Sciences) Initiative for Minority Student Development to Baylor College of Medicine, and the Howard Hughes Medical Institute (HHMI) and the Robert A. Welch Foundation (to D.J.M.).

20 February 2002; accepted 23 April 2002
Published online 2 March 2002;
10.1126/science.1072891
Include this information when citing this paper.

Operant Reward Learning in *Aplysia*: Neuronal Correlates and Mechanisms

Björn Brembs,* Fred D. Lorenzetti,* Fredy D. Reyes, Douglas A. Baxter, John H. Byrne†

Operant conditioning is a form of associative learning through which an animal learns about the consequences of its behavior. Here, we report an appetitive operant conditioning procedure in *Aplysia* that induces long-term memory. Biophysical changes that accompanied the memory were found in an identified neuron (cell B51) that is considered critical for the expression of behavior that was rewarded. Similar cellular changes in B51 were produced by contingent reinforcement of B51 with dopamine in a single-cell analog of the operant procedure. These findings allow for the detailed analysis of the cellular and molecular processes underlying operant conditioning.

Learning about relations between stimuli [i.e., classical conditioning (*1*)] and learning about the consequences of one's own behavior [i.e., operant conditioning (*2*)] constitute the major part of our predictive understanding of the world. Although the neuronal mechanisms underlying appetitive and aversive classical conditioning are well studied (e.g., *3–8*), a comparable understanding of operant conditioning is still lacking. Published reports include invertebrate aversive conditioning (e.g., *9–12*) and vertebrate operant reward learning (e.g., *13*). In several forms of learning, dopamine appears to be a key neurotransmitter involved in reward (e.g., *14*). Previous research on dopamine-mediated operant reward learning in *Aplysia* was limited to in vitro analogs (*15–18*). In this report, we overcome this limitation by developing both in vivo and single-cell operant procedures and describe biophysical correlates of the operant memory.

The in vivo operant reward learning paradigm was developed using the consummatory phase (i.e., biting) of feeding behavior in *Aplysia*. This model system has several features that we hoped to exploit. The behavior occurs in an all-or-nothing manner and is thus easily quantified (see supplemental video). The circuitry of the underlying central pattern generator (CPG) in the buccal ganglia is well characterized (*19*). The anterior branch of the esophageal nerve

Department of Neurobiology and Anatomy, W. M. Keck Center for the Neurobiology of Learning and Memory, The University of Texas–Houston Medical School, Houston, TX 77030, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: john.h.byrne@uth.tmc.edu

(En_2) (Fig. 1A) is both necessary and sufficient for effective reinforcement during in vivo classical conditioning and in vitro analogs of classical and operant conditioning (*15–18*, *20–23*). Presumably, En_2 conveys information about the presence of food during ingestive behavior. Consequently, we investigated the role of En_2 in the reinforcement pathway by recording from it in freely behaving *Aplysia* via chronically implanted extracellular hook-electrodes (*24*) (see supplemental methods) (Fig. 1A). Little nerve activity was observed during spontaneous biting in the absence of food (Fig. 1, B1), whereas bouts (duration: ~3 s) of high-frequency (~30 Hz) activity in En_2 were recorded during the ingestion of food (Fig. 1, B2). Specifically, this activity was observed in conjunction with ingestion movements of the odontophore/radula (a tonguelike organ). Electrical stimulation of En_2 might thus be used to substitute for food reinforcement in an operant conditioning paradigm. Therefore, in vivo stimulation of En_2 at approximately the frequency and duration as observed during feeding was made contingent upon each spontaneous bite in freely behaving animals (see supplemental methods). Such a preparation is unique among studies of learning in invertebrates and analogous to commonly used self-stimulation procedures in rats (e.g., *13*).

One day after implanting the electrodes, animals were assigned to one of three groups: (i) a control group without any stimulation, (ii) a contingent reinforcement group for which each bite during training was followed by En_2 stimulation, or (iii) a yoked control group that received the same sequence of stimulations as the contingent group, but the sequence was uncorrelated with their behavior (*25*). Animals that had been contingently reinforced showed significantly more spontaneous bites during a 5-min test period than did both control groups, regardless of whether they were tested immediately after training (Fig. 1C) or 24 hours later (Fig. 1D). These results indicate that during 10 min of contingent stimulation, the animals acquired an operant memory that lasted for at least 24 hours.

We next sought to identify changes in the nervous system that were associated with the behavioral modification. The neural activity that underlies the radula movements during feeding is generated by the buccal CPG. This neural network consists of sensory, inter-, and motor neurons that continue to produce buccal motor patterns (BMPs), even when the ganglia are removed from the animal (*15*). In the intact animal, ingestion-like BMPs correspond to radula movements transporting food through the buccal mass into the foregut, as opposed to rejection-like BMPs that correspond to radula movements that remove inedible objects from the foregut (*24*). Buccal neuron B51 is pivotal

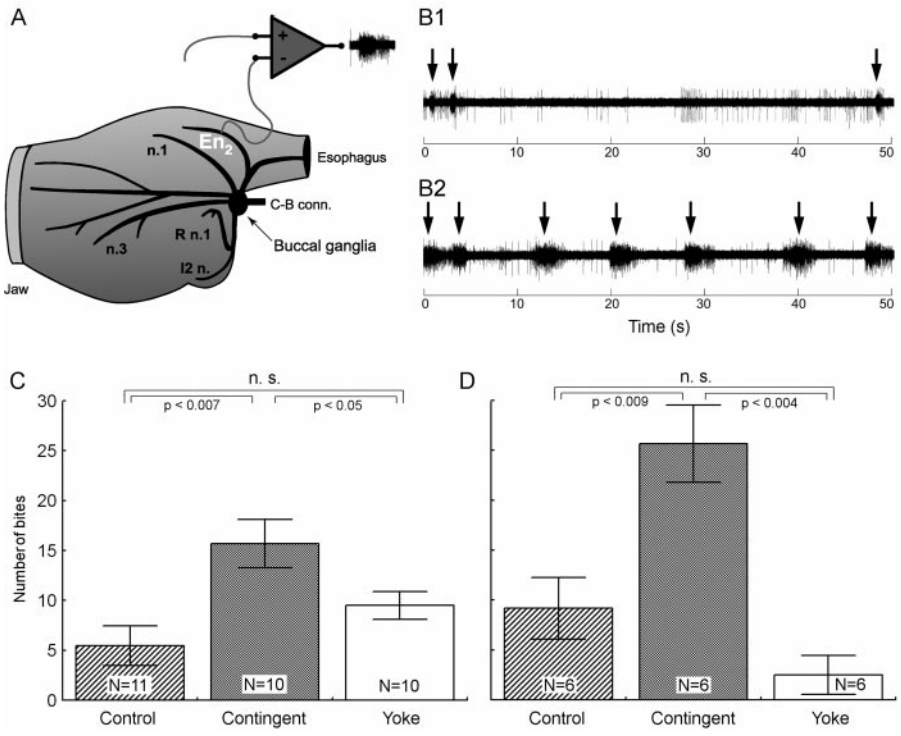


Fig. 1. In vivo recordings and behavioral results. (**A**) Schematic representation of electrode placement. (**B1**) Activity in En_2 during spontaneous bites in the absence of food. Depicted are three bites (arrows). (**B2**) Activity in En_2 during biting and swallowing behavior in the presence of food. Seven bite-swallows are shown (arrows). (**C** and **D**) Behavioral results. (C) Spontaneous bite rate in the final unreinforced test phase immediately after training. There was a significant difference among the three groups (Kruskal-Wallis ANOVA, $H_2 = 9.678$, $p < 0.008$). A post-hoc analysis revealed that the number of bites in the contingently reinforced group was significantly higher than both control and yoked groups (Mann-Whitney *U* tests, $U = 16.5$, $p < 0.007$, and $U = 24.0$, $p < 0.05$, respectively). The two control groups did not differ significantly (Mann-Whitney *U* test, $U = 29.0$, $p = 0.07$). (D) Spontaneous bite rate in the unreinforced test phase 24 hours after the beginning of the experiment. There was a significant difference among the three groups (Kruskal-Wallis ANOVA, $H_2 = 11.9$, $p < 0.003$). The number of bites taken by the contingent reinforcement group was higher than the two control groups (Mann-Whitney *U* tests, $U = 1.5$, $p < 0.009$, control; and $U = 0.0$, $p < 0.004$, yoke). The two control were not significantly different (Mann-Whitney *U* test, $U = 9.5$, $p = 0.17$). In this and subsequent illustrations, bar graphs display means ± S.E.M.

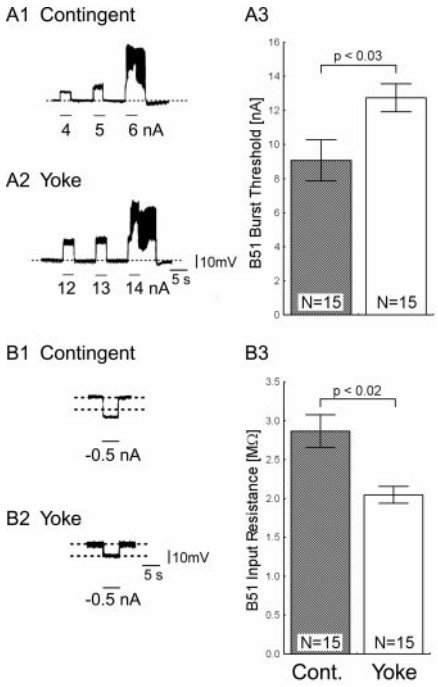


Fig. 2. Changes in burst threshold and input resistance in B51 after operant training. (**A**) Burst threshold. (A1) and (A2) Intracellular recordings from B51 cells from a matched pair of contingently reinforced and yoked control animals. Depolarizing current pulses were injected into each B51 until the cell generated a plateau potential. In this example, a 6-nA current pulse was sufficient to generate a plateau potential in B51 from a contingently reinforced animal (A1), whereas 14 nA were required to generate a plateau potential in B51 from the corresponding yoked-control animal (A2). (A3) Summary data. B51 cells from the contingent reinforcement group required significantly less current to elicit the plateau potential (Mann-Whitney *U* test, $U = 59.5$, $p < 0.03$). (**B**) Input resistance. (B1) and (B2) Intracellular recordings from B51 cells from both contingently reinforced and yoked control animals. Hyperpolarizing current pulses were injected into B51 and the cells' input resistance was measured. In this example, the membrane potential of B51 from a contingently trained animal (B1) deflected more in response to the current pulse than the potential of B51 from a yoked control animal (B2). (B3) Summary data. B51 input resistance was significantly increased in contingently reinforced animals (Mann-Whitney *U* test, $U = 37.0$, $p < 0.002$).

for the selection of BMPs. Specifically, B51 exhibits a characteristic, sustained, all-or-nothing level of activity (plateau potential) during ingestion-like BMPs. Moreover, B51 can gate transitions between BMPs. Direct depolarization of B51 leads to the production of ingestion-like BMPs, whereas hyperpolarization inhibits ingestion-like BMPs (*18*). We thus examined whether the observed increase in number of bites was associated with an increase in excitability of B51.

To test the hypothesis that B51 was a site of memory storage for operant conditioning, another set of animals was conditioned (*26*). Immediately after the last training period, the animals were anaesthetized and dissected, and the buccal ganglia were prepared for intracellular recording (see supplemental methods). Resting membrane potential, input resistance, and burst threshold were measured in B51. Burst threshold was defined as the amount of depolarizing current needed to elicit a plateau potential [see also (*16*, *18*)]. Cells from the contingent group exhibited a significant decrease in burst threshold (Fig. 2A) and a significant increase in input resistance (Fig. 2B), as compared to cells from the yoked control. The resting membrane potential did not differ among the groups (*27*). The decrease in burst threshold and increased input resistance both increase the probability of B51 becoming active and thus increase the probability that a BMP will become ingestion-like. Our data validate an in vitro analog of operant conditioning in isolated buccal ganglia (*16*) and extend the research to include operant conditioning in freely moving *Aplysia*.

Although the expression of intrinsic changes in the membrane properties of B51 was associated with operant conditioning, the maintenance of these changes could be due to extrinsic factors such as a tonic change in modulatory input to B51. If so, the locus of the associative neuronal mechanism may be upstream of B51. Moreover, as B51 is active during ingestion-like BMPs, the changes in B51 could be the effect of repeated activation, rather than a cause of operantly conditioned animals taking more bites than do the yoked control animals. To solve this question, we isolated the neuron in primary cell culture and developed a single-cell analog of the operant procedure. B51 neurons were removed from naïve *Aplysia* and cultured (see supplemental methods). Dopamine mediates reinforcement in an in vitro analog of operant conditioning (*17*), and En_2 is rich in dopamine-containing processes (*28*). Therefore, reinforcement was mimicked by a brief (6 s) iontophoretic "puff" of dopamine onto the neuron. Because B51 exhibits a plateau potential during each ingestion-like BMP, this reinforcement was made contingent upon a plateau potential elicited by injection of a brief depolarizing current pulse. Contingent reinforcement of such B51 activity in the ganglion with En_2 stimulation is sufficient for in vitro operant conditioning (*18*). Two experimental groups were examined. Building on the experience with in vitro operant conditioning (*18*), we administered seven supra-threshold current pulses in a 10-min period to a contingent reinforcement group. Dopamine was iontophoresed immediately after cessation of the plateau potential. An unpaired group received the same number of depolarizations and puffs of dopamine, but dopamine iontophoresis was delayed by 40 s after the plateau potential. Contingent application of dopamine produced a significant decrease in burst threshold (Fig. 3A) and a significant increase in input resistance (Fig. 3B). Apparently, processes intrinsic to B51 are responsible for the induction and maintenance of the biophysical changes associated with operant reward learning.

The combination of rewarding a simple behavior with physiologically realistic, in vivo stimulation uncovered neuron B51 as one site where operant behavior and reward converge (see supplemental discussion). The results presented here suggest that intrinsic cell-wide plasticity contributes to operant reward learning. Such cell-wide plasticity is also associated with operant conditioning in insects (*10*). Although B51 is a key element in the neural circuit for feeding, the quantitative contribution of the changes in B51 to the expression of the behavioral changes needs to be elucidated. Given the number of neurons in the feeding CPG (*19*), it is likely that B51 will not be the only site of plasticity during operant conditioning (nor will cell-wide plasticity likely be the only mechanism). However, the persistent involvement of contingent-dependent cell-wide plasticity in B51 in different levels of successively reduced preparations suggests an important role for this mechanism.

Research on *Aplysia* has provided key insights into mechanisms of aversive conditioning that are evolutionary conserved. The utility of this model system for learning and memory has now been extended to dopamine-mediated reward learning on the behavioral, network, and cellular level. Our study expands a growing body of literature that shows that dopamine is an evolutionary conserved transmitter used in reward systems. Future research on *Aplysia* will likely provide insights into the subcellular effects of dopamine reward, an area currently under intense investigation in vertebrates (*8*, *13*).

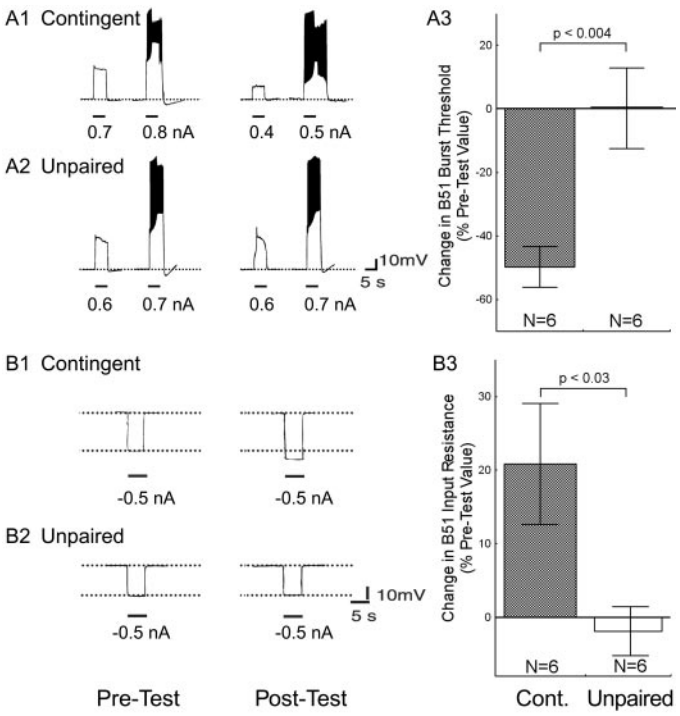


Fig. 3. Contingent-dependent changes in burst threshold and input resistance in cultured B51. (**A**) Burst threshold. (A1) and (A2) Intracellular recordings from a pair of contingently reinforced and unpaired neurons. Depolarizing current pulses were injected into B51 before (pre-test) and after (post-test) training. In this example, contingent reinforcement led to a decrease in burst threshold from 0.8 to 0.5 nA (A1), whereas it remained at 0.7 nA in the corresponding unpaired cell (A2). (A3) Summary data. The contingently reinforced cells had significantly decreased burst thresholds (Mann-Whitney *U* test, $U = 0.0$, $p < 0.004$). (**B**) Input Resistance. (B1) and (B2) Intracellular recordings from a pair of contingently reinforced and unpaired control neurons. Hyperpolarizing current pulses were injected into B51 before (pre-test) and after (post-test) training. In this example, contingent reinforcement lead to an increased deflection of the B51 membrane potential in response to the current pulse (B1), whereas the deflection remained constant in the corresponding unpaired cell (B2). (B3) Summary data. The contingently reinforced cells had significantly increased input resistances (Mann-Whitney *U* test, $U = 3.5$, $p < 0.03$).

References and Notes

1. I. P. Pavlov, *Conditioned Reflexes* (Oxford University Press, Oxford, 1927).
2. B. F. Skinner, *The Behavior of Organisms* (Appleton, New York, 1938).
3. E. T. Walters, J. H. Byrne, *Science* **219**, 405 (1983).
4. R. D. Hawkins, T. W. Abrams, T. J. Carew, E. R. Kandel, *Science* **219**, 400 (1983).
5. M. Hammer, *Nature* **366**, 59 (1993).
6. J. J. Kim, D. J. Krupa, R. F. Thompson, *Science* **279**, 570 (1998).
7. T. Zars, M. Fischer, R. Schulz, M. Heisenberg, *Science* **288**, 672 (2000).

8. P. Waelti, A. Dickinson, W. Schultz, *Nature* **412**, 43 (2001).
9. P. R. Benjamin, K. Staras, G. Kemenes, *Learn. Mem.* **7**, 124 (2000).
10. G. Hoyle, *Trends Neurosci.* **2**, 153 (1979).
11. D. Botzer, S. Markovich, A. J. Susswein, *Learn. Mem.* **5**, 204 (1998).
12. D. G. Cook, T. J. Carew, *J. Neurosci.* **9**, 3115 (1989).
13. J. N. J. Reynolds, B. I. Hyland, J. R. Wickens, *Nature* **413**, 67 (2001).
14. W. Schultz, *Nature Rev. Neurosci.* **1**, 199 (2000).
15. R. Nargeot, D. A. Baxter, J. H. Byrne, *J. Neurosci.* **17**, 8093 (1997).
16. ———, *J. Neurosci.* **19**, 2247 (1999).
17. R. Nargeot, D. A. Baxter, G. W. Patterson, J. H. Byrne, *J. Neurophysiol.* **81**, 1983 (1999).
18. R. Nargeot, D. A. Baxter, J. H. Byrne, *J. Neurosci.* **19**, 2261 (1999).
19. E. C. Cropper, K. R. Weiss, *Curr. Opin. Neurobiol.* **6**, 833 (1996).
20. H. A. Lechner, D. A. Baxter, J. H. Byrne, *J. Neurosci.* **20**, 3369 (2000).
21. ———, *J. Neurosci.* **20**, 3377 (2000).
22. M. Schwarz, A. J. Susswein, *J. Neurosci.* **6**, 1528 (1986).
23. R. Mozzachiodi, H. Lechner, D. Baxter, J. Byrne, paper presented at the 31st Annual Meeting of the Society for Neuroscience, San Diego, CA, 13 November 2001.
24. D. W. Morton, H. J. Chiel, *J. Comp. Physiol. A* **172**, 17 (1993).
25. A Kruskal-Wallis analysis of variance (ANOVA) determined that the number of bites did not differ among the three groups during an initial 5-min pretest period without reinforcement (control, 13.1 bites; contingent, 10.5 bites; yoke, 15.1 bites; $H_2 = 2.306$, $p = 0.32$, $N = 49$). Differences in bite frequency among the groups began to emerge during training. Biting increased during training in the contingent, but not in the other groups. A repeated-measures ANOVA over the two training periods (tr_1, tr_2) and the three groups yielded a significant interaction of within- and between-groups factors (control tr_1, 13.0 bites; control tr_2, 9.6 bites; contingent tr_1, 11.4 bites; contingent tr_2, 15.1 bites; yoke tr_1, 11.9 bites; yoke tr_2, 10.2 bites; $F(2, 46) = 7.198$, $p < 0.002$, $N = 49$). After training, learning performance was assessed in a 5-min test period without reinforcement.
26. In the conditioning experiment conducted to search for correlates of the operant memory in B51, an additional 5-min training period replaced the last test, to minimize extinction and ensure a high level of conditioning. Because unstimulated and yoked control groups did not differ significantly in the previous experiment, only two groups were used: contingent reinforcement and yoked control. Comparisons of the number of bites taken during the last 5-min training period assessed the success of the operant conditioning procedure. Confirming the previous results, contingently reinforced animals took significantly more bites in the last training period than did animals in the yoked control group: Mean contingent, 13.5; mean yoke, 8.4; Mann-Whitney *U* test, $U = 62.0$, $p < 0.04$.
27. Mean contingent, −65.7 mV, $N = 13$; mean yoke, −65.3 mV, $N = 12$; Mann-Whitney *U* test, $U = 77.0$, $p < 0.96$.
28. E. A. Kabotyanski, D. A. Baxter, J. H. Byrne, *J. Neurophysiol.* **79**, 605 (1998).
29. We thank E. Antzoulatos for helpful discussions and E. Wilkinson for invaluable technical assistance. B.B. is a scholar of the Emmy-Noether Programm of the Deutsche Forschungsgemeinschaft. Supported by NIH grant MH 58321.

Supporting Online Material
www.sciencemag.org/cgi/content/full/296/5573/1706/DC1
Materials and Methods
SOM Text
Fig. S1 and S2
References and Notes
Movies S1 and S2

28 December 2001; accepted 26 March 2002

Anterior Cingulate: Single Neuronal Signals Related to Degree of Reward Expectancy

Munetaka Shidara[1]* and Barry J. Richmond[2]

As monkeys perform schedules containing several trials with a visual cue indicating reward proximity, their error rates decrease as the number of remaining trials decreases, suggesting that their motivation and/or reward expectancy increases as the reward approaches. About one-third of single neurons recorded in the anterior cingulate cortex of monkeys during these reward schedules had responses that progressively changed strength with reward expectancy, an effect that disappeared when the cue was random. Alterations of this progression could be the basis for the changes from normal that are reported in anterior cingulate population activity for obsessive-compulsive disorder and drug abuse, conditions characterized by disturbances in reward expectancy.

During normal activity, we continually compare our current status against our expectation for reaching a goal, with expectation increasing over the course of the activity. That implies that there are neural signals underlying this increasing expectation.

Over the past several years, we have used visually cued multitrial reward schedules in monkeys. In this task monkeys change their error rates according to reward expectancy (*1*–*4*). To obtain a reward, monkeys must successfully complete a set (or schedule) of visual color-discrimination trials (Fig. 1A) [(*2*); see (*5*) for details of experimental procedures]. In the schedule task, the monkey has to complete between one and four color-discrimination trials successfully to obtain the reward (Fig. 1B). An unsuccessful trial is not explicitly punished, but the monkey only progresses to the next stage of a schedule when a trial is completed successfully. A second set of visual stimuli used as cues indicate progress of the schedule. The cues become brighter as the schedule progresses (cued condition). The only information available about the schedule and trial is provided by the cue. As in all of the previous studies making use of this task (*5*), the monkeys here made progressively fewer errors as the rewarded trial approached, with the fewest errors occurring in the rewarded trials (Fig. 2A), showing that the cue is actually being used by the monkey to regulate its behavior. When we randomized the cues with respect to the schedule so that the cues were no longer related to the schedule (random condition) (*5*), the monkey's error rate was always low, regardless of cue brightness (Fig. 2B). Thus, there is a substantial behavioral difference between knowing for certain what will happen in each successfully completed trial (cued condition) versus knowing the overall reward rate without knowing the outcome of each trial for certain (random condition).

For neurons in ventral striatum (*2*) and perirhinal cortex (*4*), responses occurred in specific trials of the reward schedules, with the response strengths being similar in all trials showing responses. The trials in which responses occurred appeared idiosyncratic. Thus, although the populations of neurons in either ventral striatum and perirhinal cortex could be used to decode progress through reward schedules, no single neuron carried a signal that varied directly with schedule progress or reward expectancy.

We hypothesized that within the brain's reward system, there should be a signal related to the degree of reward expectancy. For several reasons, the anterior cingulate cortex (*6*–*10*) seemed a promising site for such a signal. It appears to have a role in performance monitoring and error detection, conflict monitoring, and response selection, all of which depend on assessing reward proximity or likelihood (*11*–*18*). Several neuronal recording studies have shown associations between sensory stimuli and the expectation of various outcomes, such as reward, or pain (*19*–*24*). Finally, in several imaging studies of patients with disturbances in motivation and reward expectation, such as obsessive-compulsive disorder and drug abuse, the anterior cingulate has shown increased activation when compared with anterior cingulate in normal subjects (*25*–*38*).

We recorded from 106 single neurons in area 24c of anterior cingulate cortex [ventral bank of anterior cingulate sulcus, a part of rostral cingulate motor area (*39*), confirmed by magnetic resonance imaging (*40*)] of monkeys performing the cued multitrial reward schedule task. A substantial number of neurons (94/106) showed selective responses during the reward schedule task. For 69 neurons, activity was idiosyncrati-

[1]Neuroscience Research Institute, National Institute of Advanced Industrial Science and Technology, 1-1-1 Umezono, Tsukuba, Ibaraki 305-8568, Japan. [2]Laboratory of Neuropsychology, National Institute of Mental Health, Bethesda, MD 20892, USA.

*To whom correspondence should be addressed. E-mail: m.shidara@aist.go.jp

Research

Extending In Vitro Conditioning in *Aplysia* to Analyze Operant and Classical Processes in the Same Preparation

Björn Brembs,[1,2] Douglas A. Baxter, and John H. Byrne

Department of Neurobiology and Anatomy, W.M. Keck Center for the Neurobiology of Learning and Memory, The University of Texas Medical School at Houston, Houston, Texas 77030, USA

Operant and classical conditioning are major processes shaping behavioral responses in all animals. Although the understanding of the mechanisms of classical conditioning has expanded significantly, the understanding of the mechanisms of operant conditioning is more limited. Recent developments in *Aplysia* are helping to narrow the gap in the level of understanding between operant and classical conditioning, and have raised the possibility of studying the neuronal processes underlying the interaction of operant and classical components in a relatively complex learning task. In the present study, we describe a first step toward realizing this goal, by developing a single in vitro preparation in which both operant and classical conditioning can be studied concurrently. The new paradigm reproduced previously published results, even under more conservative and homogenous selection criteria and tonic stimulation regime. Moreover, the observed learning was resistant to delay, shortening, and signaling of reinforcement.

Ambulatory animals continuously face changing environmental situations. However, not all events are random occurrences. Some events are direct consequences either of the behavior of the animal or of some other events in the environment. If the nonrandom events are significant, animals that can predict them will have a strong adaptive advantage. Some of the most regular predictive relationships are inborn (e.g., reflexes), but many others are learned. Operant or instrumental conditioning is a form of learning in which an animal learns the predictive relationship between behaviors and the environment (Thorndike 1911; Skinner 1938), whereas classical or Pavlovian conditioning is a form of learning in which an animal learns the relationship between two environmental events (Pavlov 1927). In freely moving animals in the wild, it can be difficult to distinguish between the two, because a feedback loop exists between the behavior of the animal and the environment. For example, a frog may discover a small moving object while foraging for prey, extend its tongue toward the object, find that the object is striped and produces a noxious sting and hence in the future avoid striped insects. This well-known example of aversive conditioning illustrates the feedback loop between behavior and stimuli. The foraging behavior led to the perception of the moving object, which in turn elicited the extension of the tongue, which in turn had the noxious sting as a consequence, which in turn led to the avoidance of striped insects by the frog. It is not clear a priori which events have been remembered by the frog. Clearly, the stripes were somehow associated with the sting (a classical association between two stimuli), but was the extension of the tongue instrumental in this association? To understand such interacting events, it is necessary to first reduce them to their operant and classical components and then join them again under controlled conditions.

[1]Present address: Institute for Neurobiology, Free University Berlin, Königin-Luise-Straße 28/30, 14195 Berlin, Germany.
[2]Corresponding author.
E-MAIL bjoern@brembs.net; FAX 49 308 385 5455.
Article published online ahead of print. Article and publication date are at http://www.learnmem.org/cgi/doi/10.1101/lm.74404.

Laboratory studies of classical conditioning have successfully interrupted the operant–classical feedback loop such that the behavior of the animal is irrelevant and the two environmental events (the conditioned stimulus, CS, which predicts the unconditioned stimulus, US) can be traced from their sensory afferents to the brain and, finally, to the point where they converge and the learning occurs (e.g., Walters and Byrne 1983; Bao et al. 1998; Hawkins et al. 1998; Kim et al. 1998; Lechner et al. 2000a,b; Schafe et al. 2001; Medina et al. 2002; Paschall and Davis 2002; Ressler et al. 2002; Antonov et al. 2003; Crow and Tian 2003; Davis et al. 2003; Epstein et al. 2003; Flynn et al. 2003; Mozzachiodi et al. 2003; Nader 2003). An analogous convergence point between operant behavior and the unconditioned stimulus (or reinforcer in the operant nomenclature) has recently been described in *Aplysia* (Nargeot et al. 1999a,b; Brembs et al. 2002).

The carefully controlled operant and classical conditioning protocols used in laboratory studies are somewhat artificial learning situations, because the closed feedback loop between behavioral outputs and sensory inputs in a freely moving animal inevitably leads to many sensory stimuli eliciting behavioral responses and many behavioral actions causing the perception of sensory stimuli, all at or near the same time. One would expect that evolutionary selection pressures would form around the natural situation in which both operant and classical predictors play their parts simultaneously, so that this situation may be more easily learned than in the separate, experimental cases (i.e., composite conditioning; Brembs 2000; Brembs and Heisenberg 2000; Heisenberg et al. 2001). On the other hand, studies from vertebrates suggest that such a combination can have various effects, depending on subtle details (Williams 1975; Williams and Heyneman 1982; Williams 1989; Williams et al. 1990; Hammerl 1993; Reed 1996, 1999, 2003; Williams 1999). Therefore, as a first step toward studying the neurobiological underpinnings of operant and classical interactions, we have designed an experimental system in which operant and classical conditioning can be investigated separately, concurrently, or sequentially and which is amenable to cellular and network analysis. We took advantage of the recent advances in operant and classical conditioning of *Aplysia* feeding behavior (Susswein and Schwarz 1983;

11:412–420 ©2004 by Cold Spring Harbor Laboratory Press ISSN 1072-0502/04; www.learnmem.org

Schwarz and Susswein 1986; Colwill et al. 1997; Nargeot et al. 1997, 1999a,b,c; Lechner et al. 2000a,b; Mozzachiodi et al. 2003) and developed a computer-supported, single *Aplysia* preparation in which operant and classical experiments can be conducted both separately and in combination.

The feeding behavior of *Aplysia* (Fig. 1) offers a useful system in which to investigate classical and operant conditioning. Recently, substantial progress has been made toward understanding the neurobiology of operant conditioning of feeding behavior in *Aplysia* (Nargeot et al. 1997, 1999a,b,c; Brembs et al. 2002; Katzoff et al. 2002) as well as toward understanding the neurobiology of classical conditioning (Lechner et al. 2000a,b; Mozzachiodi et al. 2003).

Given the greater accessibility for neurobiological research, we chose to work in vitro, with reduced preparations of the *Aplysia* CNS, similar to the two previously developed in our laboratory. One in vitro preparation has been developed to study operant conditioning and another to study classical conditioning (Nargeot et al. 1997; Mozzachiodi et al. 2003). These preparations are rather similar. For example, in both, patterned motor outputs (buccal motor patterns, BMPs) are recorded extracellularly from the peripheral nerves of the buccal ganglia. This patterned activity can be interpreted as the commands for the movements of the radula/odontophore (a tongue-like organ), which lead to ingestion (or rejection) behavior (i.e., fictive feeding behavior, Fig. 1). Ingestion behavior can be classically and operantly conditioned in vivo (Susswein et al. 1983; Susswein et al. 1986; Lechner et al. 2000b; Brembs et al. 2002). The esophageal nerve (En_2) conveys the US (Schwarz and Susswein 1986; Nargeot et al. 1997; Lechner et al. 2000b; Brembs et al. 2002; Mozzachiodi et al. 2003) and the anterior tentacle nerve (AT_4) conveys the CS (Lechner et al. 2000a,b; Mozzachiodi et al. 2003). In the analog of classical conditioning the CS and US are delivered as electrical stimulation of these nerves. Thus, in behavioral terms, the BMPs constitute the operant behavior (ingestion or rejection; Morton and Chiel 1993a,b; Nargeot et al. 1997) and extracellular stimulations of the aforementioned nerves constitute the environmental feedback (i.e., stimulation of AT_4 simulates tactile stimulation of the lips; Lechner et al. 2000a,b; Mozzachiodi et al. 2003; stimulation of En_2 simulates food reward, Brembs et al. 2002).

However, besides the training protocol (operant vs. classical), there is one major difference between the two preparations. The preparation for classical conditioning included the cerebral ganglion, because it mediates the CS pathway (Lechner et al. 2000a,b; Mozzachiodi et al. 2003), whereas the operant procedure did not (Nargeot et al. 1997, 1999a,b,c).

Thus, to be able to study the interaction of operant and classical conditioning, we developed a single buccal/cerebral preparation in which classical and operant conditioning experiments can be conducted and the results compared. Moreover, this preparation will allow for the concurrent presentation of classical and operant predictors, and thereby provide a preparation that is suitable for cellular analyses of composite learning. As part of this study, we also developed a computer-assisted neuronal pattern recognition system to identify the BMPs. Most stimulation parameters were entirely computer controlled. The new preparation reproduced the previously published operant learning. Various parameter modifications indicated that the in vitro conditioning was rather robust.

RESULTS

The first step toward developing a preparation in which the interaction of classical conditioning and operant conditioning can be analyzed was to determine whether in vitro operant conditioning is expressed in the preparation originally developed to study classical conditioning (Mozzachiodi et al. 2003). Specifically, we subjected a preparation consisting of the isolated cerebral ganglion and buccal ganglion to the in vitro protocol of Nargeot et al. (1997) and investigated the extent to which the preparation reproduced the previous results. The cerebral ganglion contains higher-order neurons that can trigger the occurrence of BMPs in the buccal ganglia (Rosen et al. 1991; Jing and Weiss 2001, 2002; Hurwitz et al. 2003). It is unknown whether it

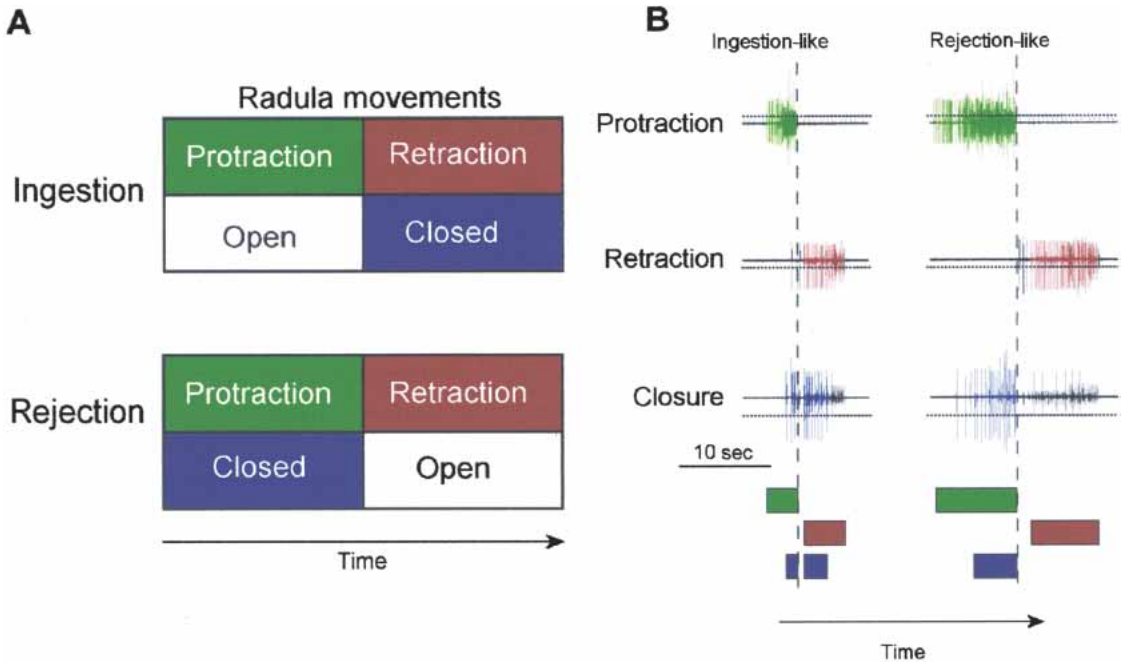


Figure 1 Pattern classification. (*A*) Schematic representation of the radula movements during ingestion and rejection. (*B*) Pattern classification deduced from the radula movements depicted in *A*. Note that only closure activity is counted that overlaps with radula movement (pro- or retraction; see Materials and Methods). Dotted lines–activity detection thresholds.

also contains cells that can silence neural activity in the buccal ganglia. Although preliminary experiments, in which we recorded from the cerebral-to-buccal connective (CBC) during spontaneous BMPs, did not reveal any evidence that spontaneous BMPs are either elicited or suppressed by signals originating in the cerebral ganglion (data not shown), the presence of either type of cell could disrupt either the occurrence of spontaneous BMPs, the ability of BMPs to be conditioned, or both.

As part of the study, we also developed a computer program (see Materials and Methods) that allowed for the control of the stimulation schedule and parameters, and to assist in distinguishing between the different types of patterns and therefore eliminate the need for a blind observer. A final aspect of the study was to vary the stimulation parameters to investigate the feasibility of experiments in which operant and classical predictors are combined.

All preparations were treated identically up until the start of the experiment, where each preparation was randomly assigned to one of six groups (Fig. 2A,B,C). These groups were designed as two triplets, the difference between the two being that one received contingent reinforcement via stimulation of the esophageal nerve and the other did not (see Materials and Methods for details; Fig. 2A,B,C). Note that some of the noncontingent groups received contingent CSs, but never contingent USs. The groups were all operant in nature and received tonic $Bn_{2,3}$ stimulation throughout the experiment. This nerve provides afferent input to the buccal ganglia. Stimulation of $Bn_{2,3}$ at a constant rate with weak intensity stimuli increases the likelihood of generating spontaneous BMPs (Nargeot et al. 1997, 1999a,b,c; Fig. 2A,B,C). Only ingestion-like BMPs (iBMPs) were reinforced.

Experimental Groups

The respective first groups in each triplet (Fig. 2A) can be seen as forming a pair designed to replicate previous studies of in vitro operant conditioning (Nargeot et al. 1997, 1999a,b), with minor parameter variations. It included a group that received a contingent US (Fig. 2A1, UScon) and a yoked control group (Fig. 2A2, USyoke). The expected outcome was an elevated number of iBMPs in the contingently reinforced compared to the yoked control group.

The respective second groups (Fig. 2B) were designed to test the effect of a delay and shortening of the reinforcing stimulus (US), as well as the effect of adding a contingent CS without a US. The contingently reinforced group (USdcon, Fig. 2B1) received a contingent US as the "UScon" group. But compared to the UScon group, the US was shortened from 6 to 4 sec and delayed by 2 sec (USdcon, Fig. 2B1). The other group (CS, Fig. 2B2) received only contingent CS presentations and no US presentations. This group was included to control for possible effects of contingent CSs alone (Fig. 2B2). The expected outcome was an elevated number of iBMPs in the USdcon group versus any of the noncontingent groups, and an unaffected number of BMPs in the group that only received a CS, compared to the other two noncontingent groups (i.e., Figs. 2A2, 1C2). Potentially, the USdcon group could have shown a lower number of BMPs than either the UScon (Fig. 2A1) or the CS+USdcon (Fig. 2C1) group.

The respective last groups in each triplet (Fig. 2C) were designed to investigate the effect of combining the shortened and delayed US with a contingent CS to "signal" the occurrence of the US (Fig. 2C). Both groups received contingent CS presentations after every iBMP, throughout the experiment. The contingently reinforced group (CS+USdcon) received contingent US presentations after each iBMP/CS combination (Fig. 2C1), whereas the control group (CS+USyoke) received the same sequence of US presentations as the contingently reinforced group, but independent of its behavior (yoked control; Fig. 2C2). In an intact *Aplysia*, the protocol of CS+USdcon would be analogous to a bite (iBMP) leading to a tactile stimulation of the lips (AT_4 stimulation) followed by food (En_2 stimulation).

Thus, in the contingently reinforced group (Fig. 2C1; CS+USdcon), during training the CS signaled the occurrence of reinforcement (US), whereas in the yoked control group (Fig. 2C2; CS+USyoke) it did not. The expected outcome is a higher number of BMPs in the contingently reinforced as compared to the yoked control. In vertebrates, such signaling can increase or decrease the amount of operant responding, depending on the choice of parameters (Williams 1975; Williams and Heyneman 1982; Williams 1989; Williams et al. 1990; Hammerl 1993; Reed 1996, 1999, 2003; Williams 1999). If a signaling effect of the CS is present in the preparation, the number of iBMPs in the CS+USdcon group is expected to be higher or lower than the number of BMPs in either the UScon or the USdcon group.

BMP Analysis

In order to assess the effects of the different treatments on the buccal Central Pattern Generator, three levels of analysis were used. First, we analyzed the total number of BMPs, irrespective of BMP-type. To gather more detailed information, we then ana-

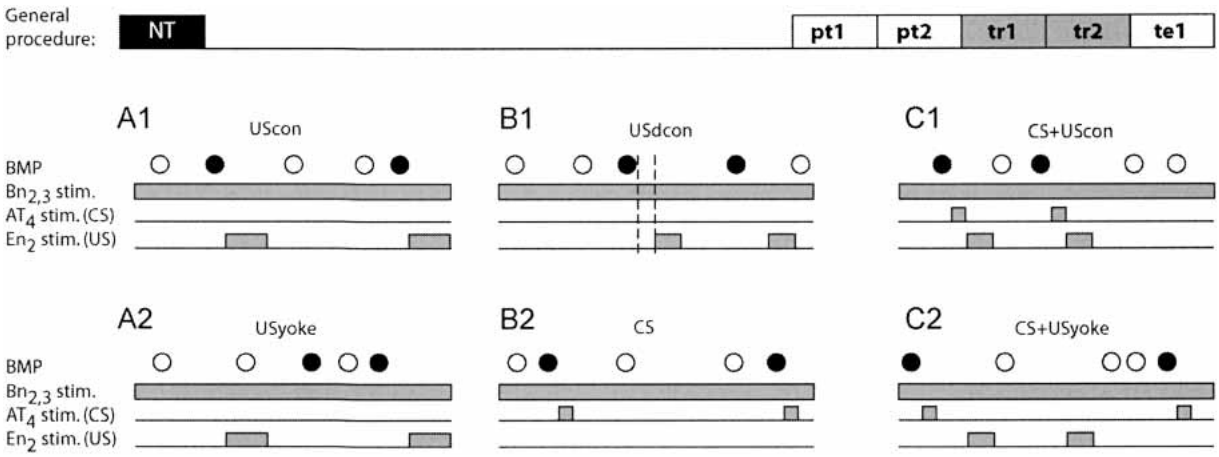


Figure 2 Schematic representation of the general procedure and the training protocols. The *top* trace illustrates the general training procedure. NT, nerve test done to establish proper conductivity of the electrodes to and from the nerves; Pt, pre-test; tr, training; te, test. The training regime for the different groups is presented schematically in *A,B,C.* Filled circles denote ingestion-like BMPs, open circles any other type of BMP.

lyzed the fraction of BMPs that were ingestion-like in nature (i.e., iBMPs). This measure has the advantage in that it describes the propensity of a preparation to produce iBMPs, irrespective of the total number of patterns produced. Finally, we evaluated the absolute number of iBMPs versus all other BMPs, to gain insight into the absolute changes in the generation of BMPs.

A one-way ANOVA (see Materials and Methods) over the total number of BMPs in all six groups did not reveal any significant variations in the total number of BMPs produced, neither in the pretest period immediately preceding the training (SS = 41.5, DF = 5, MS = 8.3, F = 0.48, p = 0.8), nor in the test immediately after the training (SS = 38.1, DF = 5, MS = 7.6, F = 0.38, p = 0.9). Thus, groups did not differ in their propensity to produce BMPs, before or after the training (i.e., treatment did not have any effect on the total number of all BMPs produced by the preparations).

Next, the fraction of iBMPs was evaluated. A one-way ANOVA over the six groups in the pretest period immediately preceding the training, was not significant (SS = 0.18; DF = 5; MS = 0.036; F = 0.74; p = 0.6). Thus, the six different groups did not differ significantly in the fraction of iBMPs produced before the training. This result indicates that all preparations had the same propensity to produce ingestion-like BMPs and any difference after training can only be attributed to the parameters of the stimulations during training.

All Contingently Reinforced Groups Increased the Propensity to Produce iBMPs

A one way ANOVA over the fraction of iBMPs in the six groups in the five minutes immediately following training, was significant (SS = 1.26; DF = 5; MS = 0.25; F = 4.5; p = 0.001). Fisher LSD post-hoc tests reveal that this significance was due to only the contingently reinforced groups differing from all noncontingent groups (Table 1). Thus, none of the different variations in US timing and duration had any effect on the magnitude of learning: contingently reinforced (via stimulation of En_2) preparations produced on average a larger fraction of iBMPs than preparations that received either no US at all or noncontingent USs, irrespective of the US parameters (Fig. 3).

Stimulation of the AT_4 nerve (such as the CS used here) can also elicit iBMPs, either after classical conditioning (Lechner et al. 2000a; Mozzachiodi et al. 2003) or if the stimulation is sufficiently intense. The application of contingent CSs in our experiments seemed to decrease (albeit insignificantly) the number of iBMPs (see Fig. 3; CS). To assess whether there was any effect from the presence or absence of the inserted CS, signaling the US, which was not uncovered by evaluating the fraction of iBMPs, a two-way repeated measures ANOVA was carried out over the absolute number of iBMPs and all other BMPs (Fig. 4). The first factor tested between contingently reinforced and noncontingent groups, whereas the second tested between pairs, and the repeated measures factor tested for differences between iBMPs and all other BMPs (Fig. 4). Only the groups with comparable US duration (4 sec) were compared, because these groups differed only in the presence or absence of the CS. Only the interaction between the repeated measures factor and the experimental/control factor was significant (SS = 92.9, DF = 1, F = 15.0, p = 0.0003; Fig. 4), meaning the distinction between experimental and control groups (i.e., the training regime) had a significant effect on the distribution of iBMPs and other BMPs among the groups. This result indicates that the presence or absence of the CS did not, but only the presence or absence of a contingency between ingestion-like BMPs and the US did have a statistically verifiable effect on the types of BMPs that were produced in the different groups. Thus, with our stimulation parameters, AT_4 stimulation by itself had no direct operant effects. The result corroborates our conclusions from the analysis of the fraction of iBMPs, namely that contingent reinforcement increases the relative number of iBMPs. In addition, a Fisher's LSD post-hoc analysis revealed that in the control groups, less ingestion-like BMPs are produced than other BMPs ($p < 0.001$) and that the number of other BMPs in the experimental groups is reduced, compared to the control groups ($p < 0.01$). Presumably because of the high value in the CS+USyoke group, the comparison of ingestion-like BMPs in experimental versus control groups fails to reach statistical significance ($p < 0.12$: see Discussion).

The limited number of preparations precludes statistically significant post-hoc differentiation between USdcon, CS+USyoke and CS+USdcon.

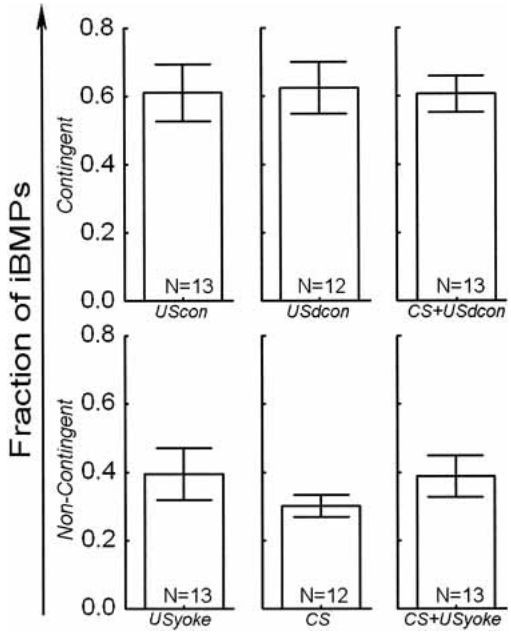


Figure 3 Frequency of ingestion-like BMPs in the six operant groups in the 5 min immediately following 10 min of training. The contingently reinforced groups showed an increased frequency of ingestion-like BMPs over the groups without contingent USs.

Table 1. p-Values for the Fisher LSD Post-hoc Tests Revealing That all Contingently Reinforced Groups Differ From All Noncontingent Groups in the Fraction if iBMPs During the Final Test Immediately Following Training

	USyoke	UScon	CS	USdcon	CS + USyoke	CS + USdcon
USyoke		0.02	0.33	0.02	0.94	0.03
UScon	0.02		0.002	0.88	0.02	0.96
CS	0.33	0.002		0.001	0.37	0.002
USdcon	0.02	0.88	0.001		0.02	0.85
CS + USyoke	0.94	0.02	0.37	0.02		0.02
CS + USdcon	0.03	0.96	0.002	0.85	0.02	

Shaded cells mark $p < 0.05$, Error: Between MS = 0.06, DF = 70.0.

DISCUSSION

We developed a computer-assisted paradigm for in vitro operant and classical conditioning in *Aplysia* that included the isolated cerebral and buccal ganglia. As a first step we investigated whether the new preparation could exhibit operant conditioning and the robustness of the operant conditioning protocol to pa-

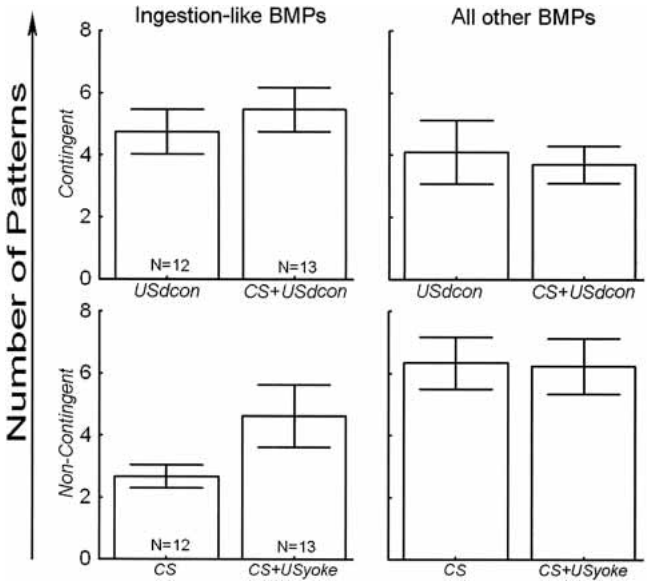


Figure 4 Absolute number of BMPs in the two pairs with a 4-sec US. The number of unrewarded patterns (i.e., noningestion-like BMPs) is reduced in the contingently reinforced groups, whereas the number of rewarded (ingestion-like) BMPs is elevated, compared to the groups that did not receive contingent USs. The high value for the CS+USyoke group may have prevented the difference in the ingestion-like BMPs from reaching statistical significance.

rameter variations including the presence of a CS signaling the reinforcer. The new paradigm reproduced previously published results, even under more conservative and homogenous selection criteria and tonic stimulation regime. Moreover, the observed learning was resistant to delay, shortening and signaling of reinforcement.

In Vitro Operant Conditioning Is Expressed in the Presence of the Cerebral Ganglion

The previous in vitro analog of operant conditioning consisted of only the isolated buccal ganglia (Nargeot et al. 1997, 1999a,b,c). It was therefore necessary to replicate the finding in a more physiological system that included the cerebral ganglion. The cerebral ganglion sends many projections to the buccal ganglion and vice versa (Rosen et al. 1991; Jing and Weiss 2001, 2002; Hurwitz et al. 2003). Therefore it was possible that the features of in vitro operant conditioning may be fundamentally different with the cerebral ganglion attached. With one exception (see below), we found that the features of operant conditioning were remarkably similar to that obtained with only the buccal ganglion. Indeed, after the six different training procedures, each contingently reinforced group produced a larger percentage of iBMPs than each group that did not receive contingent USs (Table 1; Fig. 3). Thus, we have successfully extended the in vitro operant conditioning procedure developed by Nargeot et al. (Nargeot et al., 1997, 1999a,b,c) to include the connected cerebral ganglion.

In Vitro Operant Conditioning With the Cerebral Ganglion is a Robust Phenomenon

We found that shortening and delaying the reinforcement by 2 sec did not disrupt the operant learning. We further found that adding a 2-sec CS between the ingestion-like BMPs and the reinforcement (US) also neither increased nor decreased the operant behavior.

Interestingly, delayed reinforcement is known from vertebrates to generally decrease the rate at which the operant behavior controlling the reinforcement is produced (e.g., Williams et al. 1990; Reed 1992a,b). In the case of in vitro operant conditioning of *Aplysia* feeding behavior this decrement due to delayed reinforcement apparently does not occur within the range of parameters used in the present study. Clearly, a sufficient delay of the US will eventually decrease the operant conditioning effect, as will a further shortening of the US. Thus, our paradigm has sufficient robustness to enable the study of US parameter variations: Slight variations in the reinforcement schedule do not completely disrupt learning.

Importantly, the presentation of a sensory signal (or operant CS; the 2-sec AT_4 stimulation) of reinforcement in the delay after a BMP and before reinforcement does not disrupt or enhance the production of ingestion-like BMPs, compared to the situation in which the US is merely delayed. This paradigm would be analogous to a behavior controlling both a predictive neutral stimulus (the CS) and a biologically relevant one (the US) at the same time. Returning to the example of a frog trying to capture a bee, extending the tongue would lead to a sting (US) by the striped bee (CS). In an intact *Aplysia*, the protocol would be analogous to a bite (ingestion-like BMP) leading to a tactile stimulation of the lips (AT_4 stimulation) followed by food (En_2 stimulation). It is easy to assume that the tactile lip stimulus may be interpreted as the food item moving, caused by the biting and swallowing movements. In both cases, the operant (the tongue extension or the bite) and the classical (the stripes of the bee or the lip stimulation) predictors can be perceived as competitors in the animal's search for a predictor of the reinforcer (Rescorla 1994) and antagonism as well as synergism may result. The fact that our choice of parameters led to neither synergism nor antagonism opens the possibility for parameter variations that can generate these effects. For example, the delay between the BMP and the US can be increased, allowing for a number of different arrangements of the CS within that delay. Because AT_4 stimulation has been shown previously to be able to function as a predictive signal (Mozzachiodi et al. 2003), the optimal choice of parameters should be able to create increments and decrements in the operant effect. The conspicuously high number of iBMPs in the CS+USyoke group (Fig. 4) may be an indication of how such an effect may manifest itself. In some preparations of the CS+USyoke group, concatenations of ingestion-like BMPs were observed, caused by contingent CSs eliciting BMPs. Without the reduced number of other BMPs in the CS+USyoke group and only the iBMPs thus enhanced, it is tempting to interpret this as a nonassociative effect of a combination of contingent CSs and noncontingent USs, particularly, since the CS+USdcon group was the only other group where such a concatenation of BMP–CS–BMP was observed. Although with our choice of parameters such effects were too weak to reach statistical significance, it seems possible that a different set of stimulation parameters could lead to a significant classical component in the CS+USdcon group, which, in turn, would lead to all these preparations exhibiting these concatenations of BMPs, while the yoked control preparations would remain at the same level. The accessibility of the preparation allows for a detailed analysis of the neuronal underpinnings of any such effects.

Thus, the operant effect described by Nargeot and colleagues is a robust, reproducible case of operant conditioning with the potential to study an even wider variety of behavior–CS–US relationships than space permits to present here.

Differences Between Previous Work

One of the results in Nargeot et al. (1997) that could not be reproduced was an increase in the total number of BMPs pro-

duced by the contingently reinforced preparations. In our experiments the experimental groups still produced more ingestion-like BMPs than the control groups, even in absolute numbers (data not shown), but the most clear-cut results were obtained when the frequency of ingestion-like BMPs was evaluated. Although we would not exclude the possibility that this effect stems from the presence of the cerebral ganglion, it could also be due to the asymmetrical selection criteria that were used in Nargeot and colleagues' work. Nargeot and colleagues discarded experimental preparations that produced less than five ingestion-like BMPs during the 10-min training period. No such selection was used for the control groups. Such a procedure may have selected animals in the experimental group that showed an increase in general BMP activity, independent of the operant conditioning. In our experiments, the same selection criteria were used for both experimental and control groups (see Materials and Methods). Because Nargeot and colleagues reinforced the first ingestion-like BMP in each contingently reinforced preparation, there were no latent inhibition effects that could have possibly reduced the ability of the circuit to be conditioned. In our experiments, the amount of pretest was fixed and any occurring ingestion-like BMPs during this time remained unreinforced. Moreover, our selection regime required three ingestion-like BMPs from the control groups as well and thus may have selected for too high a number of ingestion-like BMPs in these groups, masking the effect of an increase in total BMPs. Thus, while Nargeot and colleagues used a proactive selection regime that may enhance any conditioning effects, our approach was more conservative. Therefore, even under our testing conditions, the associative conditioning effect found by Nargeot and colleagues could be reproduced, emphasizing the robustness of the paradigm.

Outlook

In the future, this in vitro operant/classical conditioning paradigm can be employed to examine such long-standing questions as whether there are any operant components even in purely classical conditioning (e.g., Gormezano and Tait 1976 and references therein) or whether classical and operant conditioning are merely two aspects of the same conditioning processes (Skinner 1935; Konorski and Miller 1937a,b; Skinner 1937; Rescorla and Solomon 1967; Trapold and Winokur 1967; Trapold et al. 1968; Trapold and Overmier 1972; Rescorla and Holland 1982; Rescorla 1990a,b, 1994; Brembs and Heisenberg 2000; Heisenberg et al. 2001; Corbit et al. 2003; Holland and Gallagher 2003; Phillips et al. 2003).

MATERIALS AND METHODS

General Methods

Aplysia californica (80–350 g) were obtained from Alacrity Marine Biological Specimens and Marinus and housed individually in perforated plastic cages, floating in aerated seawater tanks at 15°C. Animals were fed ~1 g of dried seaweed three times a week. To help ensure that all animals were in a similar motivational state, experimental animals were food deprived 3–5 d before the dissection.

Dissection

Prior to dissection, the motivational state of all animals was enhanced by first feeding them a small piece of dried seaweed (~1.5 cm^2) and 30 min later a larger (8-cm^2) piece. While the animal was feeding on the larger piece, it was anaesthetized by an injection of isotonic $MgCl_2$ equivalent to 50% of its body mass. The dissection follows the procedure described in Nargeot et al. (1997, 1999a,b,c): An incision was made along the midline of the foot to expose the buccal mass and the esophagus. The most medial-ventral branch (designated branch 4) of the right anterior tentacle nerve (AT, for nomenclature, see Jahan-Parwar and Fredman 1976), which terminates in the lip region of the animal, was retained. All other peripheral nerves of the cerebral ganglion were cut short. The esophagus and the buccal mass together with the cerebral and buccal ganglia were removed and transferred to a chamber containing artificial seawater with a high concentration of divalent cations (high divalent ASW) composed of (in mM): NaCl 210, KCl 10, $MgCl_2$ 145, $MgSO_4$ 20, $CaCl_2$ 33, and HEPES 10 (pH adjusted to 7.5 with NaOH). The high divalent ASW was used to decrease neural activity during further dissection (Byrne et al. 1978). Selected peripheral nerves of the buccal ganglion were retained for extracellular recording and stimulation. The cerebral and the buccal ganglia were then pinned to the bottom of a petri dish coated with silicone elastomer (Sylgard, Dow Corning). In all experiments, the connective tissue sheath that covers the ganglia was left intact. The temperature of the static bath was maintained at 15°C with a feedback-controlled Peltier cooling device (Model SE 5010, Marlow Industries). The high divalent ASW was exchanged for normal ASW for 30 min prior to the beginning of an experiment, once the extracellular electrodes for both stimulation and recording were in place and tested for connectivity (see below). The normal ASW was composed of (in mM): NaCl 450, KCl 10, $MgCl_2$ 30, $MgSO_4$ 20, $CaCl_2$ 10, and HEPES 10 (pH adjusted to 7.5 with NaOH).

Extracellular Nerve Recordings

Previous in vivo recordings indicate that bursts of large-unit activity in nerves I_2n,Rn_1 and $Bn_{2,1}$ are associated with the protraction, closure, and retraction, respectively, of the radula/odontophore during feeding (Morton and Chiel 1993b; Hurwitz et al. 1996). Moreover, in vitro recordings indicate that BMPs, which represent fictive feeding, can be recorded from I_2n, Rn_1, and $Bn_{2,1}$ (Morton and Chiel 1993a; Nargeot et al. 1997; Lechner et al. 2000a). Thus, fictive feeding (i.e., BMPs) was monitored by placing silver electrodes on nerves I_2n,Rn_1, and $Bn_{2,1}$ (Nargeot et al. 1997) of the right buccal ganglion (see below). All extracellular electrodes were isolated from the surrounding bath using petroleum jelly (Vaseline, Sherwood Medical). Signals were amplified with a differential AC amplifier (Model 1700, A-M Systems). The amplified signals were displayed on a computer screen and saved on the hard drive using a PCI 9112 A/D converter card (Adlink Technology, Inc.) and custom-written software.

Extracellular Nerve Stimulation

Similar to our previous studies (Nargeot et al. 1997, 1999a,b,c; Brembs et al. 2002), electrical stimulation (4–6 sec, 10 Hz, 0.5-msec pulses, 7 V) of the right En_2, which innervates the buccal mass (Schwarz and Susswein, 1986) was used to mimic food reward. The duration and frequency of the stimulus resembled bursts of activity recorded in vivo from En_2 during feeding (Brembs et al. 2002). En_2 mediates several aspects of feeding behavior such as conveying efferent activity that controls peristaltic movements of the gut (Lloyd et al. 1988) and conveying afferent activity that encodes information related to feeding arousal (Susswein et al. 1984) and satiety (Kuslansky et al. 1978, 1987). Stimulation of En_2 has been used as a reinforcer to modify behavior and neural activity in a training paradigm used for operant conditioning of *Aplysia* feeding behavior both in vivo (Brembs et al. 2002) and in vitro (Nargeot et al. 1997) and in classical conditioning (Mozzachiodi et al. 2003). Moreover, En_2 is necessary for classical conditioning of feeding behavior in vivo (Lechner et al. 2000b). Finally, En_2 is necessary in an operant paradigm for learning that food is inedible (Susswein and Schwarz 1983; Schwarz and Susswein 1986). Thus, En_2 appears to be part of the reinforcement pathway that contributes to both classical and operant conditioning.

Electrical stimulation of AT_4 (2 sec, 5 Hz, 0.5-msec pulses) was used to mimic the CS that was used in classical conditioning in vivo (Lechner et al. 2000a,b) and in vitro (Mozzachiodi et al. 2003). The frequency of AT_4 stimulation used in the present study was similar to that recorded in vivo during mechanical

stimulation of the tentacles (Anderson 1967; Fredman and Jahan-Parwar 1980). The AT nerve mediates several aspects of feeding behavior. For example, AT conveys afferent activity that encodes information about both mechanical and chemical stimuli that signal the presence of food on the lips (Anderson 1967; Rosen et al. 1979; Xin et al. 1995). In addition, AT conveys efferent activity that controls the movement of the lips (Perrins and Weiss 1996). Several lines of evidence suggest that AT_4 also mediates aspects of the tactile CS that was used for in vivo classical conditioning (Lechner et al. 2000a,b). Finally, Lechner et al. (2000a) found that in vivo classical conditioning (1) increased the probability that a weak stimulation of AT_4 would elicit BMPs, and (2) enhanced the AT_4-elicited synaptic input to B31/32 in cerebral and buccal ganglia dissected from trained animals.

Following Nargeot et al. (1997), tonic stimulation of the ventral branch of buccal nerve $Bn_{2,3}$ (2 Hz, 0.5-msec pulses, 7 V) was used to nonspecifically elevate the number of spontaneous BMPs produced by the preparation.

Pulses for extracellular nerve stimulation were generated by a digital pulse generator (Pulsemaster A300, WPI) and applied, via a stimulus isolator (A360; WPI, Sarasota, FL), to bipolar silver electrodes that were placed on nerves $Bn_{2,3}$, AT_4, and En_2 and isolated from the bath with Vaseline.

Once the extracellular electrodes were in place, the high divalent ASW was exchanged for normal ASW. Preparations were washed with 50 ml ASW and then single stimulations were applied to each of the three nerves to verify electrode connectivity. Pilot studies showed that due to the high incidence of BMPs immediately after the tonic stimulation of $Bn_{2,3}$ was switched on, it was impossible to determine the appropriate sub-threshold AT_4 intensity during $Bn_{2,3}$ stimulation. Therefore, the intensity was empirically set to 3 V for all operant preparations, an intensity that on its own did not increase the number of BMPs in the pilot studies.

Classifications of BMPs

The feeding CPG expresses BMPs, which can be associated with ingestion or rejection of food (Morton and Chiel 1993a,b). BMPs consist of specific patterns of neural activity, which correspond to cycles of protraction and retraction of the radula/odontophore. BMPs can be recorded from the buccal nerves I_2n,Rn_1, and $Bn_{2,1}$. Large-unit activity in I_2n (i.e., radula protraction) precedes large-unit activity in $Bn_{2,1}$ (i.e., radula retraction). Large-unit activity in Rn_1 (i.e., radula closure) overlaps to a varying extent with protraction and retraction activity (Cropper et al. 1990; Morton and Chiel 1993a,b; Nargeot et al. 1997; Kabotyanski et al. 2000). The large-unit activity in Rn_1 corresponds to action potentials in the radula closure motor neuron B8, which has an axon in Rn_1 (Morton and Chiel 1993b; Nargeot et al. 1999b).

As in previous studies (Morton and Chiel 1993a,b; Nargeot et al. 1997; Lechner et al. 2000a; Jing and Weiss 2001, 2002; Mozzachiodi et al. 2003), we classified BMPs as ingestion-like if $\geq$50% of radula closure (Rn_1) activity occurred after the termination of the protraction (I_2n) activity. The criterion for rejection-like BMPs was the occurrence of closure (Rn_1) activity during the protraction (I_2n) activity, but no overlap between closure (Rn_1) and retraction ($Bn_{2,1}$) activity. BMPs that did not meet either of these two criteria were classified as other BMPs (Nargeot et al. 1997; Lechner et al. 2000a).

In the present study, only patterns that consisted of activity in all three buccal nerves clustered in a complete protraction/retraction cycle were classified as BMPs. Patterns consisting of bursts of activity in only one or two of the three nerves were classified as incomplete patterns and were not included in the study.

Computer-Assisted BMP Recognition

The custom-written software provided computer-assisted pattern recognition (i.e., the computer attempted an online classification and suggested a pattern type at the end of each BMP). The software was written on a MS Windows based PC using C++ and the provided software development kit for the PCI 9112 converter card. The acquisition rate was limited by processor speed, in our case to ~8 kHz. The experimenter then determined whether to follow the suggested classification or not. In the 30-min rest period, spontaneous BMPs were used to individually adjust spike detection threshold and maximal inter-spike-interval for each nerve to the individual BMPs of the experimental animal. Using these two parameters, the computer then detected "activity" in the three nerves (i.e., more than two spikes over the threshold and within the given inter-spike-interval) and correlated the timing of activity in the nerves according to the rules above. A colored line along the baseline of the recordings denoted the detected pattern type. BMP classification is usually unequivocal (Nargeot et al. 1997), but in the few ambiguous cases where radula closure activity is divided almost equally between protraction and retraction, the computer can make the objective classification much faster than the human eye.

Procedures for In Vitro Training

The procedures were based on the in vitro operant conditioning experiment developed by Nargeot et al. (1997, 1999a,b,c) and on the in vitro classical conditioning procedure developed by Mozzachiodi et al. (Lechner et al. 2000a; Mozzachiodi et al. 2003). Unlike the cited operant experiments, our preparations were given a fixed 30-min rest period without any stimulation after the connectivity of all electrodes was determined. After the rest period, two 5-min pretest periods followed, which were followed immediately by two 5-min training periods, similar to the in vivo experiments in Brembs et al. (2002). The experiment concluded with a 5-min test period, which immediately followed training. USs were only delivered to the preparation during training periods. Tonic stimulation and, where applicable, CS delivery was performed throughout the experiment. The CS presentation regime was kept constant throughout the experiment, so that only the application of the US would differentiate between training and test.

Animals were divided randomly in six groups. Each group received tonic stimulation of $Bn_{2,3}$, which began after the 30-min rest period and continued uninterrupted until the experiment ended. The groups differed from each other by the application regime of CS and US applications.

The first two groups were designed to replicate previous findings (Nargeot et al. 1997) with the difference that the cerebral ganglion was attached to the preparation. During the training period, the UScon group received contingent reinforcement (operant US deliveries) consisting of a 6-sec stimulation of En_2 immediately following each ingestion-like BMP. The corresponding USyoke group received the same sequence of En_2 stimulations during training, but uncorrelated with the occurrence of any BMPs ("yoked" control).

The third group was designed to test for the effect of a delay and shortening of the US (USdcon). This group received a contingent 4-sec US with a 2-sec delay after each ingestion-like BMP produced during training.

The fourth group was designed to test the effect of introducing contingent CSs after each iBMP without a US. This group (CS) received contingent 2-sec AT_4 stimulations (operant CSs) immediately after each ingestion-like BMP throughout the experiment and no USs during the training period.

The last two groups were designed to test the effects of introducing a signal of the delayed US. Both groups received contingent 2-sec AT_4 stimulations (operant CSs) immediately after each ingestion-like BMP throughout the experiment, starting after the 30-min rest period. During training, the CS+USdcon group received contingent reinforcement (operant 4-sec USs) immediately upon cessation of the operant CS after each ingestion-like BMP. Thus, each ingestion-like BMP in this group was followed first by a CS and then by a US; both stimulations together yielded a total of 6 sec of stimulation after each ingestion-like BMP (the US in Nargeot and colleagues original experiment had been 6 sec as well). The CS+USyoke group received the same sequence of 4-sec En_2 stimulations during the training period as

the CS+USdcon group, but uncorrelated with either generated BMPs or received CSs (yoked control).

Preparations that did not produce at least one ingestion-like BMP during training and at least three ingestion-like BMPs in the entire experiment were discarded.

Statistics

One-way or multifactor Analyses of Variance (ANOVAs) were carried out to estimate the significance of within- and between-group differences. Fisher LSD Post-hoc tests were used to detect the significant contributions to the variance in the data.

ACKNOWLEDGMENTS

We thank R. Mozzachiodi for helpful comments on an earlier draft of the manuscript. Supported by an Emmy-Noether fellowship (B.B.) and NIH Research Grant R01 MH58423 (J.H.B.).

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

REFERENCES

Anderson, J.A. 1967. Patterns of response of neurons in the cerebral ganglion of *Aplysia californica*. *Exp. Neurol.* **19:** 65–77.

Antonov, I., Antonova, I., Kandel, E.R., and Hawkins, R.D. 2003. Activity-dependent presynaptic facilitation and hebbian ltp are both required and interact during classical conditioning in *Aplysia*. *Neuron* **37:** 135–147.

Bao, J.X., Kandel, E.R., and Hawkins, R.D. 1998. Involvement of presynaptic and postsynaptic mechanisms in a cellular analog of classical conditioning at *Aplysia* sensory-motor neuron synapses in isolated cell culture. *J. Neurosci.* **18:** 458–466.

Brembs, B. 2000. An analysis of associative conditioning in *Drosophila* at the flight simulator. Ph.D. thesis, University of Würzburg, Germany. http://opus.bibliothek.uni-wuerzburg.de/opus/volltexte/2002/103/pdf/diss.pdf

Brembs, B. and Heisenberg, M. 2000. The operant and the classical in conditioned orientation in *Drosophila melanogaster* at the flight simulator. *Learn. Mem.* **7:** 104–115.

Brembs, B., Lorenzetti, F.D., Reyes, F.D., Baxter, D.A., and Byrne, J.H. 2002. Operant reward learning in *Aplysia*: Neuronal correlates and mechanisms. *Science* **296:** 1706–1709.

Byrne, J.H., Castellucci, V.F., and Kandel, E.R. 1978. Contribution of individual mechanoreceptor sensory neurons to defensive gill-withdrawal reflex in *Aplysia*. *J. Neurophysiol.* **41:** 418–431.

Colwill, R., Goodrum, K., and Martin, A. 1997. Pavlovian appetitive discriminative conditioning in *Aplysia californica*. *Anim Learn. Behav.* **25:** 268–276.

Corbit, L.H., Muir, J.L., and Balleine, B.W. 2003. Lesions of mediodorsal thalamus and anterior thalamic nuclei produce dissociable effects on instrumental conditioning in rats. *Eur. J. Neurosci.* **18:** 1286–1294.

Cropper, E.C., Kupfermann, I., and Weiss, K.R. 1990. Differential firing patterns of the peptide-containing cholinergic motor neurons b15 and b16 during feeding behavior in *Aplysia*. *Brain Res.* **522:** 176–179.

Crow, T. and Tian, L.M. 2003. Neural correlates of Pavlovian conditioning in components of the neural network supporting ciliary locomotion in *Hermissenda*. *Learn. Mem.* **10:** 209–216.

Davis, M., Walker, D.L., and Myers, K.M. 2003. Role of the amygdala in fear extinction measured with potentiated startle. *Ann. N.Y. Acad. Sci.* **985:** 218–232.

Epstein, H.T., Child, F.M., Kuzirian, A.M., and Alkon, D.L. 2003. Time windows for effects of protein synthesis inhibitors on Pavlovian conditioning in *Hermissenda*: Behavioral aspects. *Neurobiol. Learn. Mem.* **79:** 127–131.

Flynn, M., Cai, Y., Baxter, D.A., and Crow, T. 2003. A computational study of the role of spike broadening in synaptic facilitation of *Hermissenda*. *J. Comput. Neurosci.* **15:** 29–41.

Fredman, S.M. and Jahan-Parwar, B. 1980. Processing of chemosensory and mechanosensory information in identifiable *Aplysia* neurons. *Comp. Biochem. Physiol. A* **66:** 25–34.

Gormezano, I. and Tait, R.W. 1976. The Pavlovian analysis of instrumental conditioning. *Pavlov. J. Biol. Sci.* **11:** 37–55.

Hammerl, M. 1993. Blocking observed in human instrumental conditioning. *Learn. Motiv.* **24:** 73–87.

Hawkins, R.D., Greene, W., and Kandel, E.R. 1998. Classical conditioning, differential conditioning, and second-order conditioning of the *Aplysia* gill-withdrawal reflex in a simplified mantle organ preparation. *Behav. Neurosci.* **112:** 636–645.

Heisenberg, M., Wolf, R., and Brembs, B. 2001. Flexibility in a single behavioral variable of *Drosophila*. *Learn. Mem.* **8:** 1–10.

Holland, P.C. and Gallagher, M. 2003. Double dissociation of the effects of lesions of basolateral and central amygdala on conditioned stimulus-potentiated feeding and Pavlovian-instrumental transfer. *Eur. J. Neurosci.* **17:** 1680–1694.

Hurwitz, I., Neustadter, D., Morton, D.W., Chiel, H.J., and Susswein, A.J. 1996. Activity patterns of the b31/b32 pattern initiators innervating the i2 muscle of the buccal mass during normal feeding movements in *Aplysia californica*. *J. Neurophysiol.* **75:** 1309–1326.

Hurwitz, I., Kupfermann, I., and Weiss, K.R. 2003. Fast synaptic connections from cbis to pattern-generating neurons in *Aplysia*: Initiation and modification of motor programs. *J. Neurophysiol.* **89:** 2120–2136.

Jahan-Parwar, B. and Fredman, S.M. 1976. Cerebral ganglion of *Aplysia*: Cellular organization and origin of nerves. *Comp. Biochem. Physiol. A* **54:** 347–357.

Jing, J. and Weiss, K.R. 2001. Neural mechanisms of motor program switching in *Aplysia*. *J. Neurosci.* **21:** 7349–7362.

———. 2002. Interneuronal basis of the generation of related but distinct motor programs in *Aplysia*: Implications for current neuronal models of vertebrate intralimb coordination. *J. Neurosci.* **22:** 6228–6238.

Kabotyanski, E.A., Baxter, D.A., Cushman, S.J., and Byrne, J.H. 2000. Modulation of fictive feeding by dopamine and serotonin in *Aplysia*. *J. Neurophysiol.* **83:** 374–392.

Katzoff, A., Ben-Gedalya, T., and Susswein, A.J. 2002. Nitric oxide is necessary for multiple memory processes after learning that a food is inedible in *Aplysia*. *J. Neurosci.* **22:** 9581–9594.

Kim, J.J., Krupa, D.J., and Thompson, R.F. 1998. Inhibitory cerebello-olivary projections and blocking effect in classical conditioning. *Science* **279:** 570–573.

Konorski, J. and Miller, S. 1937a. On two types of conditioned reflex. *J. Gen. Psychol.* **16:** 264–272.

———. 1937b. Further remarks on two types of conditioned reflex. *J. Gen. Psychol.* **17:** 405–407.

Kuslansky, B., Weiss, K.R., and Kupfermann, I. 1978. A neural pathway mediating satiation of feeding behavior in *Aplysia*. *Behav. Biol.* **23:** 230–237.

Kuslansky, B., Weiss, K.R., and Kupfermann, I. 1987. Mechanisms underlying satiation of feeding behavior of the mollusc *Aplysia*. *Behav. Neural Biol.* **48:** 278–303.

Lechner, H.A., Baxter, D.A., and Byrne, J.H. 2000a. Classical conditioning of feeding in *Aplysia*: II. Neurophysiological correlates. *J. Neurosci.* **20:** 3377–3386.

———. 2000b. Classical conditioning of feeding in *Aplysia*: I. Behavioral analysis. *J. Neurosci.* **20:** 3369–3376.

Lloyd, P.E., Kupfermann, I., and Weiss, K.R. 1988. Central peptidergic neurons regulate gut motility in *Aplysia*. *J. Neurophysiol.* **59:** 1613–1626.

Medina, J.F., Christopher Repa, J., Mauk, M.D., and LeDoux, J.E. 2002. Parallels between cerebellum- and amygdala-dependent conditioning. *Nat. Rev. Neurosci.* **3:** 122–131.

Morton, D.W. and Chiel, H.J. 1993a. In vivo buccal nerve activity that distinguishes ingestion from rejection can be used to predict behavioral transitions in *Aplysia*. *J. Comp. Physiol. A* **172:** 17–32.

———. 1993b. The timing of activity in motor neurons that produce radula movements distinguishes ingestion from rejection in *Aplysia*. *J. Comp. Physiol. A* **173:** 519–536.

Mozzachiodi, R., Lechner, H., Baxter, D., and Byrne, J. 2003. An in vitro analogue of classical conditioning of feeding behavior in *Aplysia*. *Learn. Mem.* **10:** 478–494.

Nader, K. 2003. Memory traces unbound. *Trends Neurosci.* **26:** 65–72.

Nargeot, R., Baxter, D.A., and Byrne, J.H. 1997. Contingent-dependent enhancement of rhythmic motor patterns: An in vitro analog of operant conditioning. *J. Neurosci.* **17:** 8093–8105.

———. 1999a. In vitro analog of operant conditioning in *Aplysia*. I. Contingent reinforcement modifies the functional dynamics of an identified neuron. *J. Neurosci.* **19:** 2247–2260.

———. 1999b. In vitro analog of operant conditioning in *Aplysia*. II. Modifications of the functional dynamics of an identified neuron contribute to motor pattern selection. *J. Neurosci.* **19:** 2261–2272.

———. 1999c. Dopaminergic synapses mediate neuronal changes in an analogue of operant conditioning. *J. Neurophysiol.* **81:** 1983–1987.

Paschall, G.Y. and Davis, M. 2002. Second-order olfactory-mediated fear-potentiated startle. *Learn. Mem.* **9:** 395–401.

Pavlov, I.P. 1927. *Conditioned reflexes*. Oxford University Press, Oxford, UK.

Perrins, R. and Weiss, K.R. 1996. A cerebral central pattern generator in *Aplysia* and its connections with buccal feeding circuitry. *J. Neurosci.* **16:** 7030–7045.

Phillips, G.D., Setzu, E., Vugler, A., and Hitchcott, P.K. 2003. Immunohistochemical assessment of mesotelencephalic dopamine activity during the acquisition and expression of Pavlovian versus instrumental behaviours. *Neuroscience* **117:** 755–767.
Reed, P. 1992a. Effect of a signaled delay between an action and outcome on human judgment of causality. *Q.J. Exp. Psychol. B* **44B:** 81–100.
———. 1992b. Signaled delay of reward—overshadowing versus sign-tracking explanations. *Learn. Motiv.* **23:** 27–42.
———. 1996. Visual reinforcement signals interfere with the effects of reinforcer magnitude manipulations. *Learn. Motiv.* **27:** 464–475.
———. 1999. Role of a stimulus filling an action-outcome delay in human judgments of causal effectiveness. *J. Exp. Psychol. Anim. Behav. Process.* **25:** 92–102.
———. 2003. The effect of signaled reinforcement on rats' fixed-interval responding. *J. Exp. Anal. Behav.* **79:** 367–382.
Rescorla, R.A. 1990a. Evidence for an association between the discriminative stimulus and the response-outcome association in instrumental learning. *J. Exp. Psychol. Anim. Behav. Process.* **16:** 326–334.
———. 1990b. The role of information about the response-outcome relation in instrumental discrimination learning. *J. Exp. Psychol. Anim. Behav. Process.* **16:** 262–270.
———. 1994. Control of instrumental performance by Pavlovian and instrumental stimuli. *J. Exp. Psychol. Anim. Behav. Process.* **20:** 44–50.
Rescorla, R.A. and Holland, P.C. 1982. Behavioral studies of associative learning in animals. *Annu. Rev. Psychol.* **33:** 265–308.
Rescorla, R.A. and Solomon, R.L. 1967. Two-process learning theory: Relationships between Pavlovian conditioning and instrumental learning. *Psychol. Rev.* **74:** 151–182.
Ressler, K.J., Paschall, G., Zhou, X.L., and Davis, M. 2002. Regulation of synaptic plasticity genes during consolidation of fear conditioning. *J. Neurosci.* **22:** 7892–7902.
Rosen, S.C., Weiss, K.R., and Kupfermann, I. 1979. Response properties and synaptic connections of mechanoafferent neurons in cerebral ganglion of *Aplysia*. *J. Neurophysiol.* **42:** 954–974.
Rosen, S.C., Teyke, T., Miller, M.W., Weiss, K.R., and Kupfermann, I. 1991. Identification and characterization of cerebral-to-buccal interneurons implicated in the control of motor programs associated with feeding in *Aplysia*. *J. Neurosci.* **11:** 3630–3655.
Schafe, G.E., Nader, K., Blair, H.T., and LeDoux, J.E. 2001. Memory consolidation of Pavlovian fear conditioning: A cellular and molecular perspective. *Trends Neurosci.* **24:** 540–546.
Schwarz, M. and Susswein, A.J. 1986. Identification of the neural pathway for reinforcement of feeding when *Aplysia* learn that food is inedible. *J. Neurosci.* **6:** 1528–1536.
Skinner, B.F. 1935. Two types of conditioned reflex and a pseudo type. *J. Gen. Psychol.* **12:** 66–77.
———. 1937. Two types of conditioned reflex: A reply to Konorski and Miller. *J. Gen. Psychol.* **16:** 272–279.
———. 1938. *The behavior of organisms*. Appleton, New York.
Susswein, A.J. and Schwarz, M. 1983. A learned change of response to inedible food in *Aplysia*. *Behav. Neural Biol.* **39:** 1–6.
Susswein, A.J., Gev, S., Feldman, E., and Markovich, S. 1983. Activity patterns and time budgeting of *Aplysia fasciata* under field and laboratory conditions. *Behav. Neural Biol.* **39:** 203–220.
Susswein, A.J., Weiss, K.R., and Kupfermann, I. 1984. Internal stimuli enhance feeding behavior in the mollusc *Aplysia*. *Behav. Neural Biol.* **41:** 90–95.
Susswein, A.J., Schwarz, M., and Feldman, E. 1986. Learned changes of feeding behavior in *Aplysia* in response to edible and inedible foods. *J. Neurosci.* **6:** 1513–1527.
Thorndike, E.L. 1911. *Animal intelligence*. Macmillan, New York.
Trapold, M.A. and Overmier, J.B. 1972. The second learning process in instrumental conditioning. In *Classical conditioning II: Current research and theory* (eds. A.H. Black and W.F. Prokasy), pp. 427–452. Appleton-Century-Crofts, New York.
Trapold, M.A. and Winokur, S. 1967. Transfer from classical conditioning and extinction to acquisition, extinction, and stimulus generalization of a positively reinforced instrumental response. *J. Exp. Psychol.* **73:** 517–525.
Trapold, M.A., Lawton, G.W., Dick, R.A., and Gross, D.M. 1968. Transfer of training from differential classical to differential instrumental conditioning. *J. Exp. Psychol.* **76:** 568–573.
Walters, E.T. and Byrne, J.H. 1983. Associative conditioning of single sensory neurons suggests a cellular mechanism for learning. *Science* **219:** 405–408.
Williams, B.A. 1975. The blocking of reinforcement control. *J. Exp. Anal. Behav.* **24:** 215–225.
———. 1989. Signal duration and suppression of operant responding by free reinforcement. *Learn. Motiv.* **20:** 335–357.
———. 1999. Associative competition in operant conditioning: Blocking the response-reinforcer association. *Psych. Bull. Rev.* **6:** 618–623.
Williams, B.A. and Heyneman, N. 1982. Multiple determinants of blocking effects on operant behavior. *Anim. Learn. Behav.* **10:** 72–76.
Williams, B.A., Preston, R.A., and DeKervor, D.E. 1990. Blocking of the response-reinforcer association additional evidence. *Learn. Motiv.* **21:** 379–398.
Xin, Y., Weiss, K.R., and Kupfermann, I. 1995. Distribution in the central nervous system of *Aplysia* of afferent fibers arising from cell bodies located in the periphery. *J. Comp. Neurol.* **359:** 627–643.

Received January 22, 2004; accepted in revised form March 20, 2004.

Available online at www.sciencedirect.com
SCIENCE DIRECT
Gene 351 (2005) 131 – 142
GENE
SECTION
FUNCTIONAL GENOMICS
www.elsevier.com/locate/gene

The Drosophila *black* enigma: The molecular and behavioural characterization of the *black*[1] mutant allele

A. Marie Phillips[a,*], Renee Smart[a,1], Roland Strauss[b,1], Björn Brembs[c], Leonard E. Kelly[a]

[a]*Department of Genetics, University of Melbourne, Parkville, Victoria 3010, Australia*
[b]*Theodor-Boveri-Institut, LS Genetik und Neurobiologie, Am Hubland, 97074 Würzburg, Germany*
[c]*Institut für Neurobiologie, Freie Universität Berlin, 14195 Berlin, Germany*

Received 20 September 2004; received in revised form 21 February 2005; accepted 14 March 2005
Available online 5 May 2005
Received by D. Finnegan

Abstract

The cuticular melanization phenotype of *black* flies is rescued by β-alanine, but β-alanine production, by aspartate decarboxylation, was reported to be normal in assays of *black* mutants, and although *black*/*Dgad2* is expressed in the lamina, the first optic ganglion, no electroretinogram (ERG) or other visual defect has been demonstrated in *black* flies. The purpose of this study was to investigate the *black* gene, and protein, in *black*[1] mutants of *Drosophila melanogaster* in order to resolve the apparent paradox of the *black* phenotype. Using *black*[1] mutant flies we show that (1) aspartate decarboxylase activity is significantly reduced in adults and at puparium formation, consistent with defects in cuticular and non-cuticular processes, (2) that the *black*[1] mutation is a frameshift, and *black*[1] flies are nulls for the black/DGAD2 protein, and (3) that behavioural experiments using Buridan's paradigm, demonstrate that *black* responds abnormally to visual cues. No ERG, or target recognition defects can be demonstrated suggesting a problem with higher order visual functions in *black* mutants.

Keywords: β-alanine; Aspartate decarboxylase; Frame-shift mutation; Electroretinogram; Buridan's paradigm; Visual behaviour

1. Introduction

Mutants with defects in the *black* gene of *Drosophila melanogaster* have been known since 1910 (see Lindsley and Zimm, 1992) but the molecular and functional defects involved are not completely understood. Research into pigmentation in *D. melanogaster* established that the *black* gene encodes an essential component of a biogenic amine pathway involved in melanization and cuticular protein cross-linking (see Wright, 1987). We have previously reported the cloning of a pyridoxal-5-phosphate, PLP-dependent decarboxylase, *Dgad2*. *Dgad2* is expressed, in the adult fly, in glial cells in the first optic ganglion (lamina), and in presumptive glia associated with nerve terminals in the tergotrochanter muscles (Phillips et al., 1993). During annotation of the *Drosophila* genome, analysis of the hybridisation of our *Dgad2* clone to a translocation strain with breakpoints in the *black* gene, (Ashburner et al., 1999) was consistent with *black* encoding DGAD2. The *black* mutants have been shown to be deficient in β-alanine, and *black* mutant larvae fed or injected with this amine developed normal pigmentation and exhibited at least partial rescue of the cuticular cross-linking defect (Hodgetts and Choi, 1974; Jacobs, 1974). In its cuticular/melanization roles, β-alanine is enzymatically conjugated to another biogenic amine, dopamine, to form *N*-β-alanyl-dopamine, NBAD. NBAD is produced by *N*-β-alanyl-dopamine synthase, the product of the *ebony* gene, and the dipeptide is proposed to have a storage/transport function, reversibly inactivating two potentially toxic amines (see Wright,

Abbreviations: ERG, electroretinogram; GAD, glutamate decarboxylase; AAD, aspartate decarboxylase; NBAD, *N*-β-alanyl-dopamine; GABA, γ-amino butyric acid; kDa, kiloDaltons; bp, base pair; SEM, standard error of the mean; ANOVA, analysis of variance; LSD, least significant difference.

* Corresponding author. Tel.: +61 3 8344 7139; fax: +61 3 8344 5139.
E-mail address: m.phillips@unimelb.edu.au (A.M. Phillips).
[1] These authors contributed equally to this work.

0378-1119/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.gene.2005.03.013

1987). *N*-β-alanyl-dopamine hydrolase, NBADH, the putative product of the *tan* locus (*tan* has not yet been cloned), hydrolyses NBAD back to the component amines. The products of the *ebony*, *black* and *tan* genes are therefore all interacting components of the same pathway.

The *ebony* and *tan* mutants also have neurological defects, specifically in the visual system (Hotta and Benzer, 1969; Heisenberg, 1971; Hovemann et al., 1998). In contrast to *tan* and *ebony*, where the on and off-transients of the electroretinogram (ERG) are missing, the ERG was reported to be normal in *black* mutants (Hotta and Benzer, 1969). Mosaic data is consistent with pre-synaptic expression of the *tan* gene in the visual system (Hotta and Benzer, 1970), while, consistent with the cellular origins of the ERG transients, *ebony* is expressed in the lamina glia (Hovemann et al., 1998). As indicated above, the *ebony* gene product, NBAD synthase, is essential for NBAD formation. Neckameyer et al. (2001) found that dopamine deprivation during the 3rd instar larval stage resulted in decreased or absent ERG transients in the adult fly, indicating a role for dopamine in normal visual system development. That NBAD synthase may, in vivo, form another di-peptide, β-alanyl histidine (carnosine) was canvassed by Hovemann et al., 1998, and more recent studies support a role in the production of β-alanyl histamine (carcinine) (Borycz et al., 2002: Richardt et al., 2003). This production of carcinine identified a potential role for β-alanine in the regulation of histaminergic transmission in the visual system. Studies show that while *ebony* specifically requires β-alanine for the amino-acyladenylation step, other amines including histamine, but not amino acids, can be conjugated to the β-alanyl component (Richardt et al., 2003). We found it surprising that, if *black* and *Dgad2* were the same gene, *black* had no demonstrable visual system defect, despite its presence in lamina glial cells (Phillips et al., 1993).

Previous studies had demonstrated only a small decrease in aspartate decarboxylation in *black* mutants (Jacobs, 1974), and *black* mutants had been proposed to be defective in the uracil metabolic pathway (see Wright, 1987; Lindsley and Zimm, 1992). In order to confirm that the *black* gene does encode an aspartate decarboxylase and to investigate some aspects of the *black* paradox regarding the visual and cuticular systems, we commenced a molecular and physiological characterisation of the $black^{1}$ mutant.

2. Materials and methods

2.1. Fly stocks and crosses

All flies used in the experiments were raised on semolina-based food at 20 °C in a room with a 12 h dark/light cycle. The $black^{1}$ strain (Stock number 227) was obtained from the Bloomington Stock Center. The Oregon-R and the w^{1118}, $ebony^{11}$, tan^{1} and tan^{2} mutant strains have been maintained in Melbourne for many years. The *tan;black, tan;ebony* and *black;ebony* double mutants were generated using various balancer stocks for the X, second and third chromosomes.

2.2. Molecular biology

The *Dgad2* cDNA clone (Acc. No: NM-57440, NM-57441) and the genomic clone, are as described previously (Phillips et al., 1993). Whole fly genomic DNA was prepared by the Rapid Phenol extraction method (Jowett, 1986). PCR reactions on this DNA were performed using standard methods, Biotech (Australia) chemicals and Taq polymerase, and commercially produced oligonucleotides (Sigma Genosys). RNA was prepared by the hot phenol/chloroform RNA extraction method (Jowett, 1986). Primers used for RT-PCR were primer-1: 5′GTTCACACGGAATCACTGT 3′primer 2: 5′GCCAGCCATCCGGCGGCAGAG 3′and primer 3: 5′GAAGATAATCAGCGGCTTCC 3′. For the primer extension experiments we used a commercial kit (Promega). Primers used were: primer 1′: 5′GCTGGCCTGCGTCGAATGC 3′ and primer 2′: 5′GGTACTGTTCCTGGTGCTG 3′. The size ladder was generated using ØX174 DNA/*Hinf1* Dephosphorylated Markers (Promega).

Sequencing of DNA products was performed by the AGRF (Australian Genome Research Facility) by gel separation of Dye-terminator reactions (Big Dye terminator RR mix, Applied Biosystems).

2.3. Protein analysis

Western analysis was performed using reduced protein extracts separated on SDS PAGE gels and transferred to nitrocellulose using a semi-dry system. All filters were stained with Ponceau stain and scanned before being blocked overnight in 5% skim milk powder in 1XTBS 0.05% NP-40. Primary and secondary antibodies were applied to the filter in blocking solution. The primary antibody used was an affinity purified anti-GAD peptide antibody raised in rabbits. The commercially produced peptide, residues 138–157, was obtained from Chiron and conjugated to pertussin toxin before injection into rabbits. Cross-reacting bands were identified using HRP conjugated goat anti-rabbit antibodies (Promega) and ECL detection kit (Amersham/Pharmacia) according to the manufacturers instructions.

2.4. Enzyme assays

The enzymes assays were conducted as previously described (Phillips et al., 1993). The H^{3}-glutamate and H^{3}-aspartate (22 Ci/mmol) were obtained from Amersham. All assays were linear up to 150 μg of added protein and measurable activity was lost on heating the extract. Glutamate conversion to GABA was linear up to 45 min under all assay conditions. A 30 min incubation was used for all GAD assays shown. Aspartate conversion to β-alanine

was linear up to 10 min, and plateaued at later times. A 5 min incubation was used for all AAD assays.

Means and SEM for replicate assays were calculated, and statistical analysis of the differences between wild-type and mutant was by Student's two-tailed *t*-test, for two sets of data with different variance, or similar variance, as appropriate.

2.5. Electrophysiology

The electroretinogram (ERG) was measured as described previously (Petrovich et al., 1993) using tungsten micro-electrodes (5 mΩ, A&M Systems). The voltage trace was digitised using a PowerLab/4S and the traces analysed using Scope software (AD Instruments).

2.6. Buridan experiments

The method used for Buridan's paradigm (Götz, 1980), is similar to that described in Strauss and Pichler (1998). A test fly with shortened wings walked freely on a circular disc (diameter 85 mm) surrounded by a water-filled moat. A light-diffusing cylindrical screen (diameter 196 mm, height 160 mm) surrounded the moat so that the disc was exactly in the center. It was illuminated from the outside by four DC-driven ring-shaped fluorescent lamps (Philips, 40 W/34 "TL"E). A test situation was established with two identical black vertical stripes shown at opposite sides on the arena wall (luminance approx. 3000 cd m^{-2}; contrast 0.93). For a fly in the center of the arena the stripes extended over viewing angles of 11° horizontally and 58° vertically. The landmarks were randomly rotated into new positions after each experiment. Experiments lasted 5 min. A black-and-white video camera monitored the motion of the fly from above (Valvo CCD design board with frame transfer chip NXA1101). The video information was processed in the non-interlaced mode by an ATVista card (Truevision) in a PC. A computer program determined the position of the fly by frame scanning at 5 Hz sampling rate. The path of the fly was

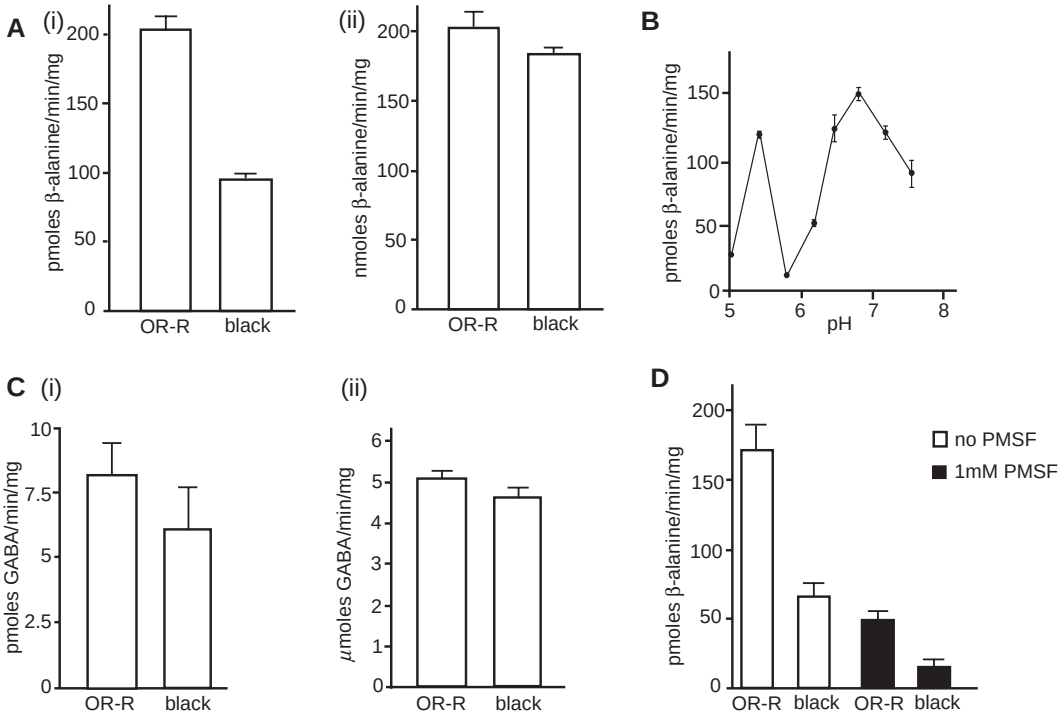


Fig. 1. Aspartate decarboxylase (AAD) activity in the *black*^{1} mutant. A: (i) Reduced AAD activity, relative to wild-type, in protein extracts from *black*^{1} mutant heads is demonstrated using an assay with 10 μM aspartate (using ^{3}H aspartate 200,000 cpm), and 100 μg protein. The protein extracts were prepared in the absence of PMSF (see D). The data shows conversion of ^{3}H aspartate to ^{3}H β-alanine in a 5 min incubation at 37 °C. The reduced activity is significantly different from wild-type (Student's two-tailed *t*-test $p=0.0028$, $n=5$). (ii) Enzyme assay as for A(i) but using 10 mM aspartate as substrate. The activity of the *black*^{1} mutant is not significantly reduced (Student's two-tailed *t*-test $p=0.35$, $n=3$). B: AAD activity in protein extracts from Oregon-R flies using the assay conditions described for A(i), but with alterations to buffer pH. For assays with pH below 5.8, 50 mM K^{+} acetate was used as buffer, between pH 5.8 and pH 7.6 the buffer was 50 mM K^{+} phosphate. The graph shows mean value and range for each point ($n=3$). Where no error bars are shown they fall within the symbol indicating the mean value. C: Glutamate decarboxylase activity (mean±SEM) in protein extracts from *black*^{1} flies relative to wild-type extracts. (i) Using 10 μM glutamate as substrate and the protein extracts used to analyse AAD activity in A, there was no significant difference in GAD activity (Student's two-tailed *t*-test, $p=0.37$, $n=5$). (ii) In experiments using 10 mM glutamate, GAD activity in mutant and wild-type is identical, as observed previously (Student's two-tailed *t*-test $p=0.88$, $n=3$). D: Conversion of aspartate to β-alanine in the presence and absence of the serine protease inhibitor phenyl-methyl-sulfonyl fluoride (PMSF). Conversion is decreased if PMSF is added to the homogenisation buffer ($p<0.015$ for both OR-R and *black* AAD assays, (Student's two-tailed *t*-test, $n=3$). Addition of PMSF immediately prior to assay also results in decreased conversion of aspartate to β-alanine. The ratio of *black*^{1} to wild-type activity is not affected.

reconstructed from the stored sequence of velocity vectors that represent direction and speed between consecutively recorded positions. All of the data shown was extracted from these recordings (Strauss and Pichler, 1998). The angle of orientation between fly and approached target was measured every 0.2 s (1500 recordings per fly). Data points with angles between 0° and 5° as well as −5° and 0°, between 5° and 10° as well as −10° and −5°, etc., were pooled and their normalized frequency plotted. The curve for random orientation was calculated as described in Strauss and Pichler (1998).

3. Results

3.1. Aspartate decarboxylase activity in black[1] flies

We had developed an assay for acidic amino-acid-decarboxylase activity (Phillips et al., 1993; Featherstone et al., 2000). In studies of flies heterozygous for deletions of the *Dgad2* locus and a *black* mutation we had not found any statistically significant reductions in glutamate/aspartate decarboxylation (Phillips et al., 1993). As genetic data and genomic sequence were consistent with the *black*

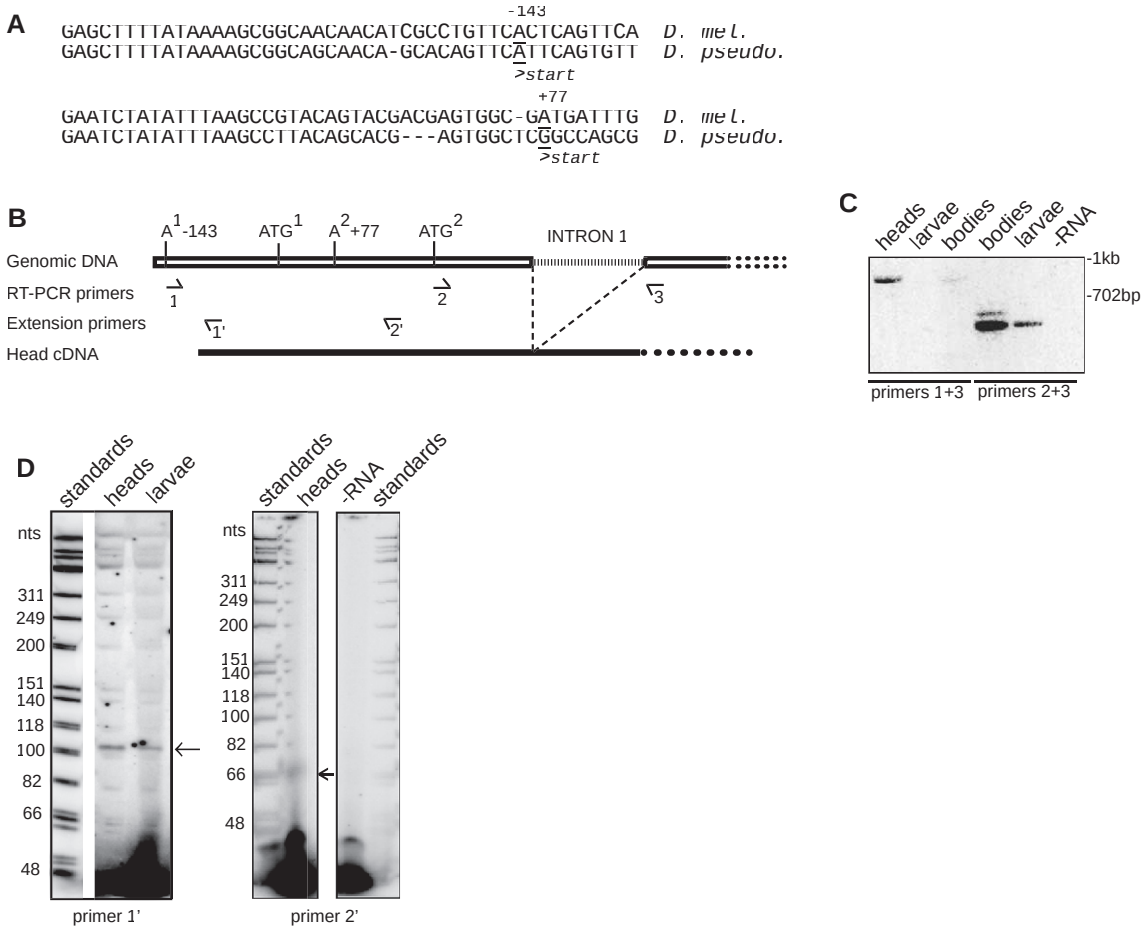


Fig. 2. Transcription from the *black/ Dgad2* locus. (A) Two putative transcription sites with equal probability values of 0.99, identified in the promoter/5′ UTR of the *black/Dgad2* gene in *D. melanogaster*, using the Berkeley Drosophila Genomes Project (BDGP) analytical tools program (Reese, 2001). *D. pseudoobscura* also has two putative start sites for both transcription (probability values 0.99 and 0.98) and translation in *black/Dgad2*. (*D. pseudoobscura* database at Baylor College of Medicine, In 12 Drosophila Genomes Resource, The FlyBase Consortium (2003). http://flybase.org/). The position, in base pairs, of the putative start sites for transcription are given relative to the first ATG. (B) Diagrammatic representation of the genomic DNA of the *black* locus showing the position of the two putative transcriptional start sites A^1 and A^2, the two translational start sites ATG^1 and ATG^2, and the position of the first intron. Primers used for the RT-PCR and the primer extensions, are indicated by the half arrows. The solid line indicates the 5′ extent of the longest cDNA found in the library screens (Phillips et al., 1993). (C) RT-PCR using RNA from heads, bodies and larvae using the primers 1, 2 and 3 shown in (B). The expected and observed sizes of the fragments were 944 bp (primers 1 and 3) and 538 bp (primers 2 and3). Equivalent amounts of RNA were used for all assays except for the body RNA using primers 1 and 3 where 10× RNA was used. Sequencing confirmed the products as deriving from *Dgad2* mRNA. (D) Primer extension experiment using RNA isolated from adult heads and larvae at puparium formation. The primers, 1′ and 2′ in (B), were labelled with [γ-^{32}P] ATP, 3000 Ci/mmol, 10 mCi/ml. Probe and standard ladder labelling incubations were for 45 min and annealing reactions were performed overnight. Extension times were for 30 min. Primer 1′ is located at −73 to −91 bp from the first translation start site. The arrow indicates the primer 1′ extension product generated from the longer transcript (to −143 bp). This product is present using RNA derived from heads and larvae. Using primer 2′, located at +163 to +180, a shorter transcript could not be unambiguously identified, although there is some indication of an extension product using head RNA (indicated by arrow).

and *DGad2* loci being synonymous, we decided to assay aspartate decarboxylase activity in homozygous *black*^{1} flies. Although separation of active GAD enzymes has been achieved by immunoprecipitation, our antibodies would not precipitate the enzyme, so we evaluated DGAD2 activity using crude protein extracts. The K_M for *Drosophila* glutamate decarboxylase had been established as 11 mM using partially purified enzyme (Chude et al., 1979). Altering the aspartate concentration in our assay from 10 mM to 10 μM revealed a significant reduction in aspartate conversion to β-alanine in *black*^{1} head protein extracts relative to wild-type (Fig. 1A(i) and (ii)) (PMSF was omitted from the buffer, see Fig. 1D). This is the first demonstration of a significant decrease in aspartate decarboxylase activity in a *black* mutant, and is consistent with DGAD1 and DGAD2 decarboxylases differing in their substrate specificity. We then used 10 μM aspartate as substrate in studies of pH effects on activity. In the studies of Chude et al. (1979), assays of DGAD activity using the crude enzyme showed two pH optima (at pH 5.0 and pH 7.2) while the partially purified DGAD enzyme had a single peak of activity at pH 7.2. Using 10 μM aspartate and crude protein extracts from heads of Oregon-R wild-type flies, two peaks of AAD activity were seen, the first peak at pH 5.4 and a second peak at pH 6.8 (Fig. 1B). At pH>8 the assays could not be evaluated as there was non-enzymatic conversion of the substrate (data not shown). All subsequent AAD assays were conducted at pH7.0, where there was a measurable difference between *black*^{1} and Oregon-R (Fig. 1A(i)) and where any small change in pH allowed reproducibility between assays. Protein extracts from heads of *black*^{1} mutant homozygotes showed a significant reduction in activity in multiple experiments using 10 μM aspartate at pH 7.0. This reduction in enzyme activity in *black*^{1} mutants is seen when aspartate, but not glutamate, is used as a substrate (Fig. 1C(i) and (ii)).

Interestingly, greater decarboxylse activity (in terms of recoverable radioactivity) could be demonstrated in protein extracts when the serine protease inhibitor, phenyl-methyl-sulfonyl-fluoride (PMSF) was omitted from the buffer during homogenization (Fig. 1D). Addition of PMSF (1 mM) to protein samples immediately prior to the assay also decreased measurable activity. However even in the presence of PMSF, the data are consistent with a significant (>50%) decrease in aspartate decarboxylase activity in the *black*^{1} mutant.

3.2. The Dgad2 transcripts

We have shown by Northern blot analysis that there are two mRNAs produced from *Dgad2* in whole adult flies (Phillips et al., 1993). On the basis of size, the head-derived cDNA (Phillips et al., 1993) must be from the larger of these

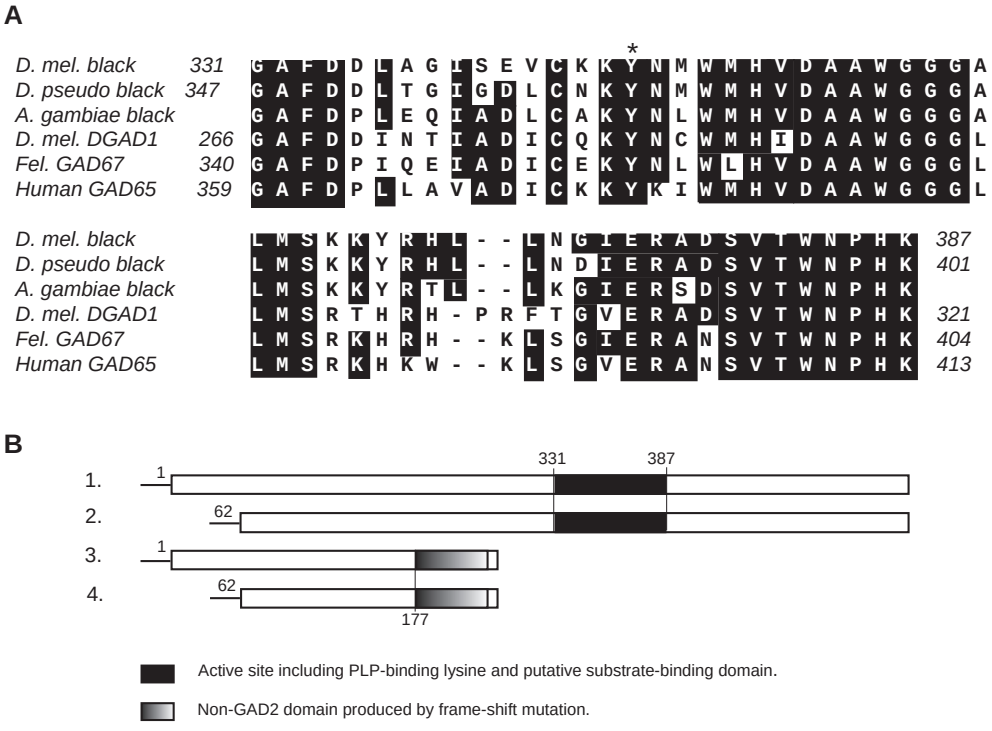


Fig. 3. Effect of the mutations in *black*^{1} on the encoded protein. (A) Alignment of the active site of *black/DGAD2* from *D. melanogaster, D. pseudoobscura* and *A. gambiae,* and of the related PLP-dependent decarboxylases, *D. melanogaster* DGAD1 (Jackson et al., 1990), *Feline* Gad67 (Kobayashi et al., 1987) and Human Gad65 (Bu et al., 1992). The asterisk identifies the conserved tyrosine, residue 347, that is altered to histidine in the *black*^{1} mutant. (B) Diagrammatic representation of the two wild-type proteins encoded by the *black/Dgad2* locus if both translation start sites are used, bars 1 and 2. The changes in these proteins resulting from the *black*^{1} frame-shift mutation at 530 bp, bars 3 and 4.

two transcripts. We considered the possibility that the two transcripts might be differentially expressed, one in the cuticle and the other in glial cells. By analogy, in a gene encoding an evolutionarily related PLP decarboxylase, *dopa-decarboxylase* (*ddc*), different transcripts are used for neuron-specific and cuticular expression (Scholnick et al., 1986). Mutations affecting DGAD2 expression in the cuticle but not the nervous system could be a possible explanation for the difference in the visual phenotype between *ebony* and *black*.

The genomic sequence was analysed for potential transcription start-sites and two with equal likelihood (0.99) were predicted by the program (Reese, 2001). The first site is at −143 bp, and the second at +77 bp from the first putative translation start codon in the cDNA. The second is therefore between the two translation start codons (Fig. 2A and B). Two similar sites are predicted in *D. pseudoobscura* (Fig. 2A) and both of the putative translational start sites are also retained. RT-PCR and sequencing confirmed that a transcript extending from the putative first start site (A^1 in Fig. 2B) is present in adult head and in adult bodies (Fig. 2C). This is in agreement with the original cDNA (Phillips et al., 1993) that extends 5′ to the A^2 start site (Fig. 2B). However using a more 3′primer (primer 2 in Fig. 2B) RT-PCR with both body and larval RNA produced a strong reaction product (Fig. 2C) suggesting that there may indeed be a shorter transcript produced. Using primer extension a product was identified in adult head RNA corresponding to initiation at A^1. A similarly sized product was observed using larval RNA (Fig. 2D−primer 1). The longer transcript must therefore be present in head, body and larvae. An extension product consistent with a shorter transcript may be present in head mRNA, however the signal is weak, and the transcript is shorter than that expected from initiation at A^2 (Fig. 2D−primer 2). While proving that the longer transcript is produced, the data does not confirm that tissue-specific differences in transcription of the *black* gene are present, nor that it might account for the *black* mutant phenotype.

3.3. Molecular analysis of the Dgad2 gene

The *black*^{1} mutation arose spontaneously (see Lindsley and Zimm, 1992) and is homozygous viable. For these reasons the molecular defect is likely to be confined to the *black* locus. Sequencing of the *black*^{1} promoter revealed many single base changes, but none affecting the transcriptional start sites. The open reading frame encoding DGAD2 was then sequenced in the *black*^{1} mutant using overlapping PCR products derived from four individual preparations of genomic DNA. In *black*^{1} the replacement of four bases (ATCC) by an eight base pair insertion (TACCTACC) at position +530 bp in the cDNA sequence results in a frame-shift. If expressed this would produce a truncated, enzymatically-inactive protein (Fig. 3B). There were also 18 single base pair substitutions in the *black*^{1} mutant sequence, compared to the cDNA sequence (Phillips et al., 1993). Of these, only one a T to C substitution at +1042 bp, resulting in a tyrosine (Y) to histidine (H) alteration at residue 348, might be functionally significant as this Y is conserved across species and in related decarboxylases (Fig. 3A).

The *Dgad2* cDNA clone, the G-2 genomic clone (Phillips et al., 1993), both derived from Canton-S, and the genomic sequence in Flybase (Scaffold No AE003641, Ashburner et al., 1999; Adams et al., 2000; Celniker et al., 2002) were identical in sequence. Laboratory strains, Oregon-R and w^{1118}, also showed conservation of the *Dgad2* sequence. Overall, the amino acid sequence in all

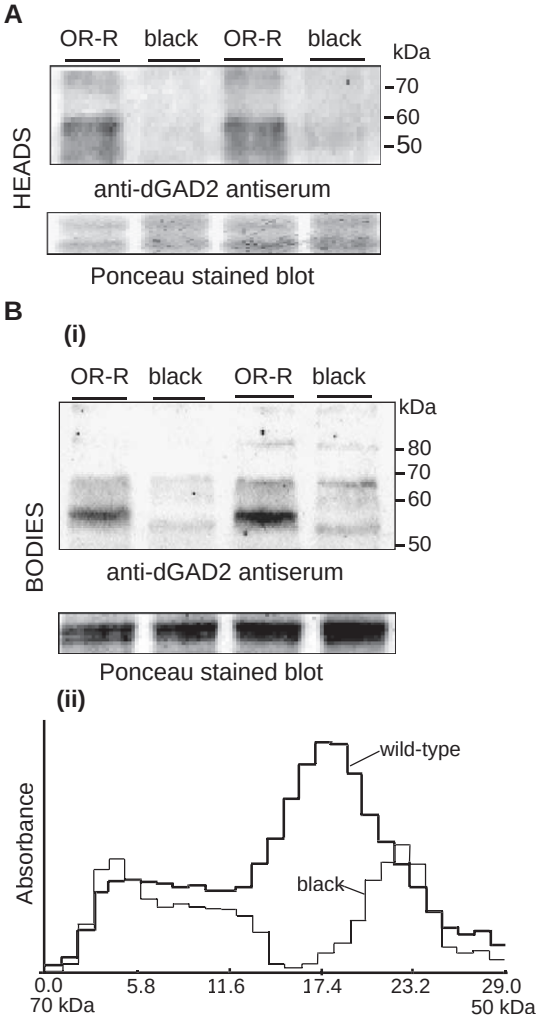


Fig. 4. The black/DGAD2 protein is absent from *black*^{1}. A: Western blots showing a protein at 58 kDa in the heads of adult wild-type flies that is not observed in the *black*^{1} mutant. The Ponceau stained panel is of the portion of the antibody probed filter, showing even loading. B: (i) Western blot of probed proteins from OR-R and *black*^{1} bodies. The data from this blot, are consistent with a 58 kDa protein being absent from *black*^{1} flies. This is confirmed in (ii) which shows the densitometric traces from the last two lanes superimposed. There is a major peak of cross reacting material present in wild-type that is missing from *black*^{1}. Other cross-reacting species are common to both genotypes.

strains studied, with the exception of *black*^{1}, shows a high level of conservation (>99% identity).

3.4. DGAD2 protein in *black*^{1}

Soluble proteins extracted from the heads or bodies of wild-type adult flies and *black*^{1} mutant homozygotes, were compared on Western blots. When probed with affinity purified anti-DGAD2 antibodies, a protein around 58 kDa is identified in wild-type but not *black*^{1} mutant extracts (Fig. 4A and B(i)). Densitometry confirmed that this protein species was missing from *black*^{1} and that the slightly lower molecular weight protein seen in *black*^{1} extracts was present, but masked by the DGAD2 signal in wild-type extracts (Fig. 4B(ii)). The first in-frame AUG in the original *Dgad2* cDNA would be expected to produce a protein of 64 kDa. The second in-frame AUG would produce a 58 kDa protein. There is no evidence on the Western blots for the larger 64 kDa protein, and extracts of heads and bodies run on the same gel show coincident mobility of the antibody reacting proteins (data not shown). No equivalent protein is produced in *black*^{1} mutant flies.

Truncated protein produced by the *black*^{1} mutant would be either 22.3 kDa or 16 kDa and would cross-react with the antibodies used in these experiments (see Fig. 3B). However in out-crossed flies no low-molecular weight proteins were seen on the Western blots that correlated with the presence of the *black*^{1} allele. In contrast the 58 kDa protein species was present in both wild-type homozygotes and heterozygous sibs, indicating the segregation of this protein with the wild-type allele. To retain any possible DGAD2 activity in *black*^{1} flies, translation would have to reinitiate at the fourth available AUG after out-of phase termination; an unlikely possibility. The *black*^{1} homozygous flies are therefore true nulls for DGAD2 and highly suitable animals for analysis of the function of the *black* locus.

3.5. Physiological assays and phenotypes

β-alanine levels increase in larvae at instar boundaries, at the larval/pupal boundary and in pupae at eclosion (Hodgetts, 1972). To determine if the expression pattern of DGAD2 fitted this profile, protein extracts were prepared from *black*^{1} and Oregon-R larvae for the three days preceding the 3rd instar/pupal boundary. Equivalent amounts of total protein from these six extracts were Western blotted and probed with anti-DGAD2 antibody. No cross-reactivity was seen associated with DGAD2 in *black*^{1} larvae/pupae, but in wild-type Oregon-R a 58 kDa band increased in intensity over the 3 day period (Fig. 5A). The change in expression pattern of DGAD2 at pupariation correlates with the changes in β-alanine observed by Hodgetts (1972). The up-regulation of DGAD2 at the larval/pupal interface may be mediated by the putative ecdysone-receptor binding consensus motif at −156 to −142 in the genomic sequence (Fig. 5B). Aspartate decarboxylase activity of *black*^{1} larvae at the larval/pupal boundary showed a 70% reduction in activity as compared with wild-type (Fig. 5C).

The expression of *Dgad2* in the first optic ganglion suggests a neuronal/visual system role for *black* (Phillips et al., 1993). However *black*^{1} mutant flies have normal ERGs, consistent with the published literature (Hotta and Benzer, 1969; Fig. 6). Extensive studies comparing *black*^{1} under both dark-adapted and ambient light conditions, and at different light intensities showed no differences from wild-type. This included *black*^{1} flies that had been outcrossed to remove any modifiers. We generated the double mutants *black*^{1}*;tan*^{1}, *black*^{1}*;tan*^{2}, *black*^{1}*;ebony*^{11} and *ebony*^{11}*;tan*^{1}. The pigmentation of the double mutant flies was consistent with that expected from the published literature. However, none of the double mutants had on- or off-transients (for example see Fig. 6). The data indicate a functional difference between the ability to produce β-alanyl-histamine

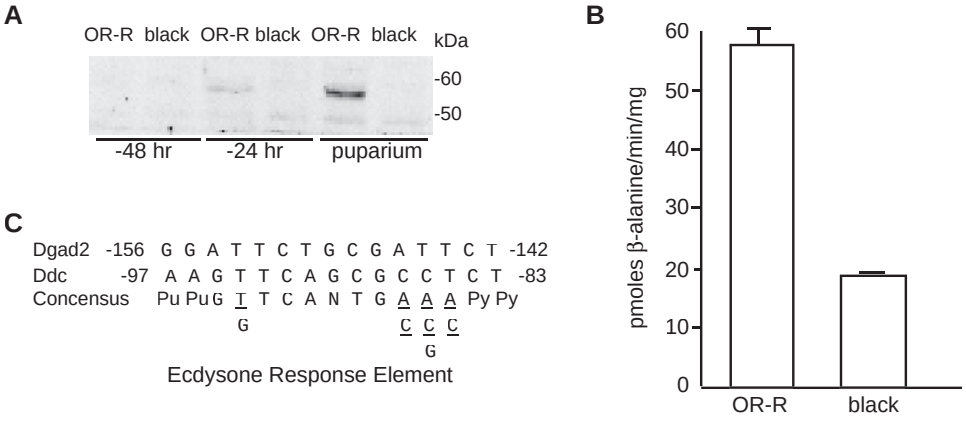


Fig. 5. DAGD2 at puparium formation. (A) Western blot, probed with affinity purified anti-black/Dgad2 antibodies, shows increasing levels of a 58 kDa protein in the 48 h up to pupation, in OR-R flies. (B) Aspartate decarboxylase assays using protein extracts from *black*^{1} and wild-type larvae at puparium show a 70% reduction in β-alanine production in the *black*^{1} mutant. The assay used 10 μM aspartate, 100 μgprotein extract and a 5 min incubation at 37 °C. (C) A putative ecdysone receptor-binding element (EcRE) is identified −156 bp to −142 bp from the most 5′ transcription start site in the *black* promoter. The *Ddc* gene has an EcRE −97 bp to −83 bp from the transcription start site (Chen et al., 2002).

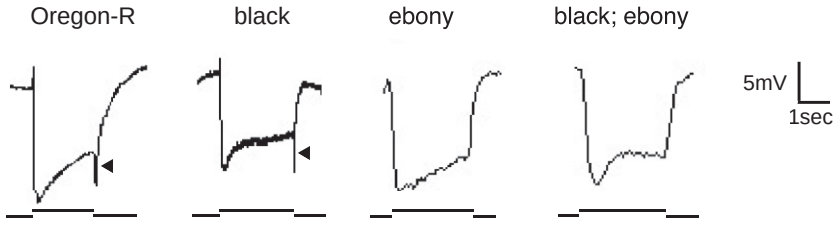


Fig. 6. Electroretinograms of the *black*^{1} mutant. Electroretinograms recorded from wild-type (Oregon-R), *black*^{1}, *ebony*, and *black*^{1}*; ebony* double mutant flies. The *ebony* flies show no transient responses at light-on or light-off indicating a failure of transmission from the photoreceptor cells to those in the first optic ganglion. In contrast the *black*^{1} flies show no effect on the transient responses. The phenotype of the *black*^{1}*; ebony* double mutant is identical to that of *ebony* flies.

(defective in *ebony*), and the ability to produce β-alanine (defective in *black*).

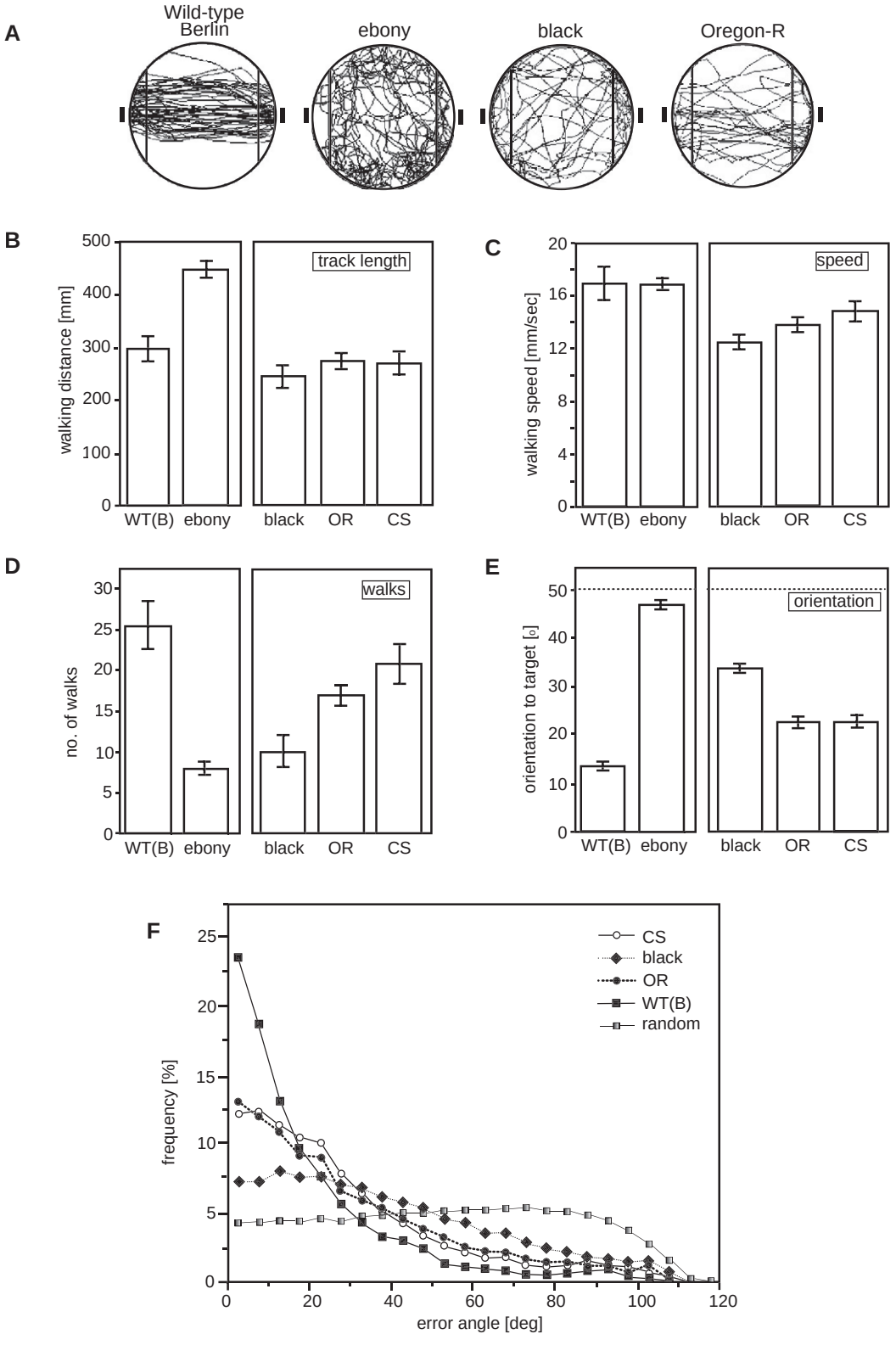
3.6. Aberrant orientation behaviour in *black*^{1} flies

In experiments using Buridan's paradigm where flies walk between two visual cues (Götz 1980), a clear difference between wild-type (Berlin, Oregon-R), *ebony* and *black* fixation behaviour was observed (Fig. 7A). These experiments confirmed the effects of the visual defects in *ebony* where any landmark fixation and walking appeared to be close to the random level expected of blind flies, or wild-type flies walking without landmarks (Fig. 7A). As the Berlin wild-type is known to perform particularly well in Buridan's paradigm, we have used two wild-type strains, Oregon-R and Canton-S for comparison with *black*. Berlin wild-type and *ebony* are used to identify the extremes of behaviour in the paradigm. From the traces of Oregon-R, Canton-S and *black*, we computed the walking distance, walking speed, number of walks and fixation (i.e. deviation from target). A Wilk's multivariate ANOVA over the four factors and the three groups was significant (F: 8.1398; df: 8; $p \ll 0.001$), allowing further analysis. Fisher LSD post hoc tests revealed no statistically significant variation in the walking distance of all flies ($p > 0.3$, Fig. 7B). Nor is there any difference in the walking speed between *black* and the two wild-type strains ($p > 0.05$ in all cases, Fig. 7C). This contrasts with the behaviour of *ebony*. The walking distance covered by the *ebony* mutants significantly exceeded those of the wild-type flies ($p < 0.001$, Fig. 7B). This is explained by missing pauses in front of the landmarks. At the same time *ebony* flies produced the least number of transitions between the counting zones in front of the landmarks (Fig. 7A and D). Due to the nature of the trails, individual walks become exceedingly long (Fig. 7B).

Thus, walking itself does not seem impaired in *black* mutants as both the walking speed and distance covered are equivalent to wild-type. However, when the number of walks initiated were compared, *black* differed significantly from both Oregon-R ($p = 0.029$) and Canton-S ($p = 0.0016$) (Fig. 7D). Analysis of fixation behaviour also showed a significant deficit in *black* flies (Fig. 7E). All of the wild-type genotypes measured in Buridan's paradigm showed fixation, although as predicted, Berlin was far stronger than either Oregon-R or Canton-S (Fig. 7E). Fixation is calculated as the mean peak frequency for angles between current path increments of a given test fly and the current direct path to one of the landmarks. To quantify fixation we calculated the error angle for which 50% of all observations fall between 0° error and this calculated angle (Fig. 7E and

Fig. 7. Analysis of Buridan's paradigm traces. (A) Examples of traces of single flies walking for 5 min between inaccessible visual landmarks (symbolized by solid bars flanking the circles). The genotypes are as indicated. The traces for *ebony* and wild-type Berlin were generated in a separate series of experiments. ($n = 11$ for *ebony* and *black*, 20 for wild-type Berlin, 16 for Oregon-R and 14 for Canton-S). (B) Mean ± SEM of the total distance walked during the 5 min experiments for each genotype. The black data are not significantly different from Oregon-R or Canton-S (see text). (C) Mean walking speed ± SEM. Mutants do not differ significantly from the wild-types (see text). (D) Mean ± SEM of the number of transitions between the landmarks (number of walks) for each genotype. Both *ebony* and *black* differ significantly from the wild-types. See text for probabilities. (E) Mean ± SEM of error angles that mark the 50% of walks that deviate the least from the target. The dotted line indicates the 50% error angle for random walking. The mutants differ significantly from the wild-types. For *black* vs. Oregon-R $p \gg 0.001$. (F) The angle of orientation between fly and approached target was measured every 0.2 s (1500 recordings per fly). Data points with angles between 0° and 5° as well as −5° and 0°, between 5° and 10° as well as −10° and −5°, etc., were pooled and their normalized frequency plotted. The curve for random orientation was calculated as described (Strauss and Pichler, 1998). The data for *black* falls between the random curve and the curves for the two wild-type strains, Oregon-R and Canton-S.

F, the area underneath the frequency curve in Fig. 7F is bisected at this value). The mean frequency distribution of the mutant *black* flies showed a broad plateau between 0° and 40° instead of an upward trend towards 0° error angle in the wild-type strains (Fig. 7F). Their fixation abilities were nevertheless clearly better than random, but significantly worse than either Oregon-R or Canton-S ($p \ll 0.001$; Fig. 7F). Both Oregon-R ($p = 0.0298$) and Canton-S ($p = 0.0016$) also differ from *black* in initiating walks (Fig. 7D). If fixation is the impetus driving the initiation of walks, then



the reduction in the number of walks initiated by *black* flies may be a reflection of the inability of *black* to fixate effectively.

4. Discussion

This study, along with mapping the *black* gene to 34C (Woodruff and Ashburner, 1979), and the in situ hybridisation data using the cDNA clone (Ashburner et al., 1999), establishes the *black* phenotype as being due to a defect in the acidic amino acid decarboxylase, DGAD2. A reduction in enzyme activity to less than 50% is seen both in $black^1$ mutant adult flies and during $black^1$ larval development. Decreased DGAD2 activity is seen when aspartate, but not glutamate, is used as a substrate. This implies that DGAD2 shows substrate specificity for aspartate, and is producing β-alanine in situ. Defects in the uracil pathway have long been proposed as the basis of the reduction in β-alanine in the *black* mutant (see Lindsley and Zimm, 1992). It is now clear that the *black* mutation is not due to a defect in the uracil pathway.

The residual activity seen in $black^1$ homozygotes is likely to represent the activity of related decarboxylases such as DGAD1. Glutamate decarboxylase enzymes are able to decarboxylate aspartate in vitro (Porter and Martin, 1988) and DGAD1 is widely expressed in the adult head (Jackson et al., 1990). Chude et al. (1979) found two pH optima of GAD activity in crude *Drosophila* extracts. We see two optima for AAD activity at similar but not identical pHs to those for GAD. The semi-purified GAD had a single pH optimum around neutral pH, which is the pH we selected for AAD assays. This supports our hypothesis that DGAD1 is producing the AAD activity seen at this pH in $black^1$ mutant extracts. It is possible that DGAD2 is decarboxylating glutamate in some cells, but that decreased glutamate decarboxylase activity in the $black^1$ mutant is masked by the presence of the more abundant DGAD1 enzyme. We would therefore not exclude the possibility that DGAD2 produces both β-alanine and, in some tissues GABA. As recombinant DGAD2 is inactive, a clear answer to this question awaits the purification of native protein.

Despite the presence of two adult mRNAs that hybridised with *Dgad2* we have been unable to definitively confirm that this derives from variation in the 5′ sequence of the *Dgad2* mRNA. Nor have we been able to show two forms of the protein. Currently our data is consistent with a single soluble DGAD2 protein of 58 kDa being produced from the *black* locus, although the transcript found to be present could produce a larger protein of 64 kDa. The protein observed may derive from a shorter, rare RNA species, as yet undetected, or be a processed form of a larger protein.

The putative GAD2 homologues in *D. pseudoobscura* and *A. gambiae* show considerable sequence identity to the *D. melanogaster* gene with *D. pseudoobscura* GAD2 having 80% identity and 97% similarity to the *D. melanogaster* protein. In *A. gambiae* identity is around 70% for sequence that is annotated although the initiating methionine and adjacent amino terminal sequences could not be identified. This conservation across dipteran species suggests that mutations affecting protein function would be detrimental. Sequencing of homozygous $black^1$ mutants revealed that $black^1$ is functionally a null for the encoded aspartate decarboxylase. At least two mutations are functionally significant, the tyrosine to histidine in a domain likely to be important in substrate recognition, and an insertion/inversion mutation resulting in a frame shift. The structural mutation resembles a transposable element footprint and although both these mutations have occurred spontaneously, it is not possible to determine which mutation was the primary event. A large number of silent changes present in the mutant may reflect the genetic background of the parental strain, or result from an accumulation of mutations in the unselected gene.

The apparently normal visual phenotype of *black* mutants has been difficult to understand given current hypotheses. β-alanine can be conjugated to histamine and the inability of *ebony* flies to form carcinine has been suggested to result in their abnormal visual function and lack of ERG transients. However *black* flies have normal ERGs. The *black* mutant flies cannot make β-alanine via the decarboxylation of aspartate and hence, like *ebony*, should be defective in carcinine production (Borycz et al., 2002). This then poses a paradox. The absence of β-alanine in the cuticular melanization pathway creates a black fly, as does the enzymatic defect in *ebony*. However this similarity between *black* and *ebony* does not extrapolate to the ERG transients despite both products being expressed in the lamina. Borycz et al. (2002) have found that histamine levels are low in both *ebony* and *black* and both are deficient in carcinine. Studies by others (McDonald and Rosbash, 2001; Richardt et al., 2003) show that while *black* mRNA cycles in response to light/dark cues, as does *histidine decarboxylase* (*hdc*) and *ebony*, the *black* message peaks some 6–7 h earlier than the other messages i.e. *black* is most highly expressed in the night and *hdc* and *ebony* at dawn. Mutations in the *tan* gene produce abnormal ERG transients, but *tan* has not been cloned, and defects in vesicle cycling, and visual system changes due to abnormal development (Neckameyer et al., 2001) may be as important as any postulated enzymatic activity in determining the ERG phenotype in *tan* mutants. A build up of free β-alanine in *ebony* flies, with a consequent inhibitory effect on the lamina response, is one possible explanation for the ERG differences between *black* and *ebony*. However this phenotype would be suppressed in a *black* mutant where β-alanine cannot be synthesised. We observe no suppression of the *ebony* ERG defect in the *black/ebony* double mutant. Overall, the evidence for a similar bio-genic amine pathway acting in both the visual system and in the cuticle of flies is not compelling.

Out-crossing the *black*^{1} flies, and chromosomal replacement, has eliminated any unlinked modifiers, unless such modifiers are common in laboratory strains. Compensatory up-regulation of either another decarboxylase or of the uracil metabolic pathway has been considered. However, from published data (Borycz et al., 2002) on carcinine levels in *black*^{1} flies, there is no evidence of an alternative source of β-alanine in the *black*^{1} visual system.

From the "Buridan's paradigm" traces, one is tempted to conclude that while *ebony* is unable to see the landmarks at all, *black* can see them, but is either unable to fixate properly or fails to see the landmarks with wild-type resolution. Motor deficits in *black* have been reported previously (Jacobs, 1978; Elens, 1965). Jacobs (1978) describes *black* walking behaviour as an "unsteady gait", and Elens (1965) found a decrease in motor activity. Our data indicate no difference in levels of walking distance or speed in *black*^{1} compared to the wild-type strains in the 5 min Buridan's paradigm. Given that these are identical, the reduced ability of *black*^{1} to fixate the two stripes is not likely to be due to a motor deficit. A deficit in *black*^{1} visual acuity is one possibility. The behavioural changes in *black*^{1} suggest it is more likely that DGAD2 is acting on higher-order visual system functions. Further studies on the visual system of *black* mutants (for instance optomotor experiments) are required to support or refute this hypothesis. It is not known why wild-type flies incessantly run from one landmark to the other only to turn around and run back an instant later. One can speculate that the fly is trying to escape the bright arena and it may well be that *black*^{1} mutants have reduced perception of this visual stimulus.

The structure of β-alanine is similar to that of glycine and GABA, the two major inhibitory neurotransmitters, and it is frequently used as an agonist/antagonist in studies of receptors and pumps. Recently, a mammalian G-protein-coupled receptor specifically responsive to β-alanine has been isolated, the first such receptor identified (Shinohara et al., 2004). There have also been much earlier reports of a direct inhibitory role for β-alanine in the vertebrate visual system (see Sandberg and Jacobson, 1981). It is tempting to speculate that β-alanine has some neuro-modulatory role in vivo. *Dgad2* expression is associated with the musculature of the fly (Phillips et al., 1993) while there is no similar expression reported for either *ebony* or *tan*. This further supports a role for β-alanine in adult *Drosophila* outside any functions associated with β-alanyl-amines.

In summary, the *Drosophila black* gene has been shown to have non-cuticular expression in the adult fly. In this paper we show that the *black*^{1} mutant is a null, and conversion of aspartate to β-alanine in protein homogenates from these flies is significantly reduced. The data are consistent with *Dgad2*/*black* encoding the aspartate decarboxylase activity required for melanization and cuticle formation. However, *black* appears not to be acting through this pathway in the visual system. Whether *black* is producing β-alanine/GABA as a neurotransmitter, or forming a dipeptide, for example with histamine, to form carcinine, and regulating excitatory activity, *black* and *ebony* mutants acting through the biogenic amine pathway should have the same phenotype. In the absence of evidence of intervening compensatory regulatory pathways we must hypothesise that *black* has no function in histamine metabolism in the lamina, or that the currently proposed pathway is incorrect or incomplete.

Acknowledgements

We are grateful for the assistance of B. Wittek and A. Baier in collecting the behavioural assay data, to J. Roote (University of Cambridge) for genetic information and flies, and to Q. Lang and S. Baxter for their computer expertise. This work was supported in part by grants from the NH & MRC and ARC to LEK.

References

Adams, M.D., et al., 2000. The genome sequence of *Drosophila melanogaster*. Science 287, 2185–2195.

Ashburner, M., et al., 1999. An exploration of the sequence of a 2.9-MB region of the genome of *Drosophila melanogaster*: the Adh region. Genetics 153, 179–219.

Borycz, J., Borycz, J.A., Loubani, M., Meinertzhagen, I.A., 2002. *tan* and *ebony* genes regulate a novel pathway for transmitter metabolism at fly photoreceptor terminals. J. Neurosci. 22, 10549–10557.

Bu, D.F., et al., 1992. Two human glutamate decarboxylases, 65-kDa GAD and 67-kDa GAD, are each encoded by a single gene. Proc. Natl. Acad. Sci. U. S. A. 89, 2115–2119.

Celniker et al., 2002. Genome Biology 3:research0079.1-0079.14.

Chen, L., Reece, C., O'Keefe, S.L., Hawryluk, G.W., Engstrom, M.M., Hodgetts, R.B., 2002. Induction of the early-late *Ddc* gene during *Drosophila* metamorphosis by the ecdysone receptor. Mech. Dev. 114, 95–107.

Chude, O., Roberts, E., Wu, J.-Y., 1979. Partial purification of *Drosophila* Glutamate Decarboxylase. J. Neurochem. 32, 1409–1415.

Elens, A.A., 1965. Studies of selective mating using the melanistic mutants of *Drosophila melanogaster*. Experientia 21, 145–146.

Featherstone, D.E., Rushton, E.M., Hildebrand-Chae, M., Phillips, A.M., Jackson, F.R., Broadie, K., 2000. Glutamic acid decarboxylase (GAD) is required for differentiation of the postsynaptic glutamate receptor field at the *Drosophila* neuromuscular junction. Neuron 27, 71–84.

FlyBase Consortium, 2003. The FlyBase database of the Drosophila genome projects and community literature. Nucleic Acids Res. 31, 172–175.

Götz, K.G., 1980. Visual guidance in *Drosophila*. In: Siddiqi, O., Babu, P., Hall, L.M., Hall, J.C. (Eds.), Development and Neurobiology of Drosophila. Plenum Press, New York, pp. 391–407.

Heisenberg, M., 1971. Separation of receptor and lamina potentials in the electroretinogram of normal and mutant *Drosophila*. J. Exp. Biol. 55, 85–100.

Hodgetts, R.B., 1972. Biochemical characterisation of mutants affecting the metabolism of β-alanine in *Drosophila*. J. Insect Physiol. 18, 937–947.

Hodgetts, R., Choi, A., 1974. Beta-alanine and cuticle maturation in *Drosophila*. Nature 252, 710–711.

Hotta, Y., Benzer, S., 1969. Abnormal electroretinograms in visual mutants of *Drosophila*. Nature 222, 354–356.

Hotta, Y., Benzer, S., 1970. Genetic dissection of the *Drosophila* nervous system by means of mosaics. Proc. Natl. Acad. Sci. 67, 1156–1163.

Hovemann, B.T., Ryseck, R.-P., Waldorf, U., Stortkuhl, K.F., Dietzel, I.D., Dessen, E., 1998. The *Drosophila ebony* gene is closely related to microbial peptide synthetases and shows specific cuticle and nervous system expression. Gene 221, 1–9.

Jackson, F.R., Newby, L.M., Kulkarni, S.J., 1990. Drosophila GABAergic systems: sequence and expression of glutamic acid decarboxylase. J. Neurochem. 54, 1068–1078.

Jacobs, M.E., 1974. β-alanine and adaptation in *Drosophila*. J. Insect Physiol. 20, 859–866.

Jacobs, M.E., 1978. β-alanine tanning of *Drosophila* cuticles and chitin. Insect Biochem. 8, 37–41.

Jowett, T., 1986. Preparation of nucleic acids. In: Roberts, D.B. (Ed.), *Drosophila* a Practical Approach. IRL Press, Oxford, UK, pp. 275–286.

Kobayashi, Y., Kaufman, D.L., Tobin, A.J., 1987. Glutamic acid decarboxylase cDNA; nucleotide sequence encoding an enzymatically active fusion protein. J. Neurosci. 727, 68–72.

Lindsley, D.L., Zimm, G.G., 1992. The Genome of *Drosophila Melanogaster*. Academic Press, San Diego, CA, USA.

McDonald, M.J., Rosbash, M., 2001. Microarray analysis and organization of circadian gene expression in *Drosophila*. Cell 107, 567–578.

Neckameyer, W., O'Donnell, J., Huang, Z., Stark, W., 2001. Dopamine and sensory tissue development in *Drosophila melanogaster*. Neurobiology 47, 280–294.

Petrovich, T.Z., Merakovsky, J., Kelly, L.E., 1993. A genetic analysis of the *stoned* locus and its interaction with *dunce, shibire*, and *Suppressor of stoned* variants of *Drosophila melanogaster*. Genetics 133, 955–965.

Phillips, A.M., Salkoff, L.B., Kelly, L.E., 1993. A neural gene from *Drosophila melanogaster* with homology to vertebrate and invertebrate glutamate decarboxylases. J. Neurochem. 61, 1291–1301.

Porter, T.G., Martin, D.L., 1988. Non-steady-state kinetics of brain glutamate decarboxylase resulting from interconversion of the apo-and holoenzyme. Biochim. Biophys. Acta 874, 235–244.

Reese, M.G., 2001. Application of a time-delay neural network to promoter annotation in the *Drosophila melanogaster* genome. Comput. Chem. 26, 51–56.

Richardt, A., Kemme, T., Wagner, S., Swarzer, D., Marahiel, M.A., Hovemann, B.T., 2003. Ebony, a nonribosomal peptide synthetase for beta-alanine conjugation with biogenic amines in *Drosophila*. J. Biol. Chem. 278, 41160–41166.

Sandberg, M., Jacobson, I., 1981. β-alanine, a possible neurotransmitter in the visual system? J. Neurochem. 37, 1353–1356.

Scholnick, S.B., Bray, S.J., Morgan, B.A., McCormick, C.A., Hirsh, J., 1986. CNS and hypoderm regulatory elements of the *Drosophila melanogaster* dopa decarboxylase gene. Science 234, 998–1002.

Shinohara, T., et al., 2004. Identification of a G-protein-coupled receptor specifically responsive to beta-alanine. J. Biol. Chem. 279, 23559–23564.

Strauss, R., Pichler, J., 1998. Persistence of orientation toward a temporarily invisible landmark in *Drosophila melanogaster*. J. Comp. Physiol., A 182, 411–423.

Woodruff, R.C., Ashburner, M., 1979. The genetics of a small autosomal region of *Drosophila melanogaster* containing the structural gene for alcohol dehydrogenase. Genetics 92, 117–132.

Wright, T.R.F., 1987. The genetics of biogenic amine metabolism, sclerotisation and melanisation in *Drosophila melanogaster*. Adv. Genet. 24, 127–222.

Research

Context and occasion setting in *Drosophila* visual learning

Björn Brembs[1,4] and Jan Wiener[2,3]

[1]*Institute of Biology, Neurobiology, Freie Universität Berlin, Königin-Luise-Strasse 28/30, 14195 Berlin, Germany;* [2]*University of Würzburg, Department of Genetics and Neurobiology, Biozentrum am Hubland, 97074 Würzburg, Germany*

In a permanently changing environment, it is by no means an easy task to distinguish potentially important events from negligible ones. Yet, to survive, every animal has to continuously face that challenge. How does the brain accomplish this feat? Building on previous work in *Drosophila melanogaster* visual learning, we have developed an experimental methodology in which combinations of visual stimuli (colors and patterns) can be arranged such that the same stimuli can either be directly predictive, indirectly predictive, or nonpredictive of punishment. Varying this relationship, we found that wild-type flies can establish different memory templates for the same contextual color cues. The colors can either leave no trace in the pattern memory template, leading to context-independent pattern memory (context generalization), or be learned as a higher-order cue indicating the nature of the pattern-heat contingency leading to context-dependent memory (occasion setting) or serve as a conditioned stimulus predicting the punishment directly (simple conditioning). In transgenic flies with compromised mushroom-body function, the sensitivity to these subtle variations is altered. Our methodology constitutes a new concept for designing learning experiments. Our findings suggest that the insect mushroom bodies stabilize visual memories against context changes and are not required for cognition-like higher-order learning.

Παντα ρει και ουδεν μενει–Everything flows, nothing stands still (Heraclitus). Rapid changes in environmental contingencies require flexible capacities through which organisms can come to expect biologically significant events (unconditioned stimuli, US) and modify the behavior in anticipation of those events if behavior is to remain adaptive; i.e., increase the probability of obtaining beneficial and avoiding harmful consequences (Sutton and Barto 1998; Dickinson and Balleine 2002). In a dynamic environment, some of the stimuli can predict the occurrence of single USs (conditioned stimuli, CS), others may indicate the nature of the CS–US contingency (occasion setters, OS) and again others may be present without having any relationship to the US whatsoever (context). Thus, in order to be able to form an accurate expectation of future USs, animals have to extract from the universe of sensory signals the actual predictors by separating them from nonpredictive stimuli. In principle, this can be achieved if only those sensory inputs that bear a temporal relationship to the reinforcer are taken as predictors (Wickens 1987).

Tethered *Drosophila* can be trained to avoid heat punishment (US). Predictors of heat punishment can be the behavior of the fly, a variety of stimuli or almost any combination of both (Wolf and Heisenberg 1991, 1997; Wolf et al. 1998; Ernst and Heisenberg 1999; Liu et al. 1999; Brembs and Heisenberg 2000, 2001; Heisenberg et al. 2001; Tang et al. 2004; Katsov and Clandinin 2006). In these paradigms, the fly is attached to a measuring device that transduces the fly's turning behavior (yaw torque) into an analog signal (Fig. 1). The signal can be used to establish any kind of behavioral consequence (Heisenberg et al. 2001). We have used the unique environmental control this set-up affords to highlight the role of the temporal relationship of initially neutral stimuli (context, CS, OS) and US, and its consequences for the acquisition of predictive memory in wild-type and transgenic flies.

Take, for instance, differential conditioning of visual patterns (Wolf and Heisenberg 1991; Ernst and Heisenberg 1999; Brembs and Heisenberg 2000; Tang et al. 2004; Katsov and Clandinin 2006). In this paradigm, animals learn to avoid one visual pattern (e.g., an upright T) and to prefer another (e.g., an inverted T). All other stimuli remain constant throughout the experiment. Slightly modifying the experiment by changing the background color between training and test (e.g., from blue-green to blue or from blue-green to green or vice versa) does not disrupt performance (Liu et al. 1999). The color remains constant during training and thus the T-patterns are the sole reliable predictors of reinforcement—the colors fulfill the definition of context. Wild-type flies can generalize the pattern memory across certain contexts (context-independent memory), while flies with impaired mushroom-body function cannot (Liu et al. 1999). The pattern memory of the mushroom-body-impaired flies is thus context-dependent. Interestingly, context dependence is often presented as a costly or advanced brain capacity or feature, while context independence is often described as a failure of the brain to incorporate the context into the memory template (e.g., Law et al. 2004). It is curious that flies with impaired mushroom-body function should exhibit such a feature, while wild-type flies fail to do so. Is context dependence a feature or a failure of the brain? One explanation for the low learning scores in the transgenic or mushroom-bodyless flies may be that they are not able to perform the separation between patterns and colors (Liu et al. 1999). In this view, the exhibited context dependence is a failure to separate patterns from colors, forming a compound memory template. A second explanation may be that the flies with compromised mushroom-body function detect much more quickly than wild-type flies that there is no punishment at all in the new situation. This enhanced detection then abolishes the pattern preference in the mushroom-bodyless flies. In this view, the context dependence of the pattern memory is a feature of the brain, not present in wild-type flies and brought about by inhibiting

[3]Present address: Collège de France, LPPA 11, Place Marcelin Berthelot, 75231 Paris.
[4]Corresponding author.
E-mail bjoern@brembs.net; fax 49-308-385-5455.
Article is online at http://www.learnmem.org/cgi/doi/10.1101/lm.318606.

13:618–628 ©2006 by Cold Spring Harbor Laboratory Press ISSN 1072-0502/06; www.learnmem.org

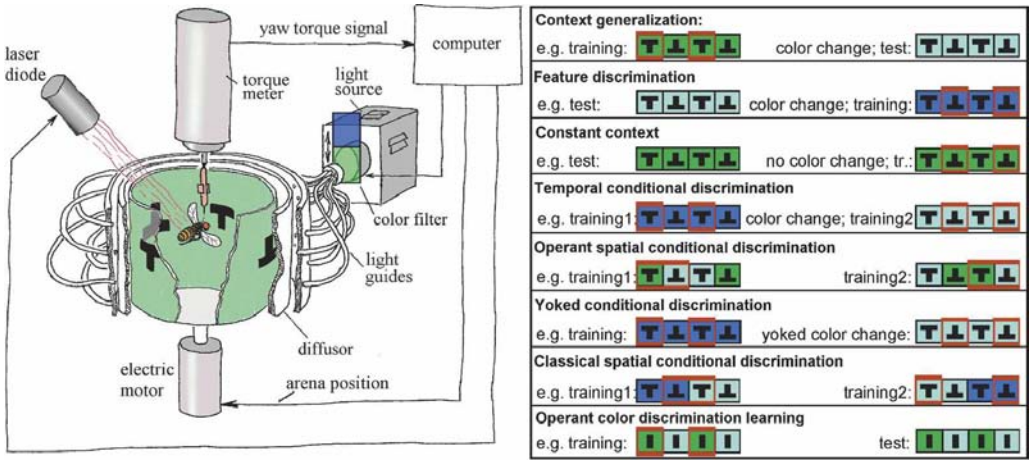


Figure 1. Flight simulator set-up and experimental schematics. (*Left*) The fly is flying stationarily in a cylindrical arena homogeneously illuminated from behind. The fly's tendency to perform *left* or *right* turns (yaw torque) is measured continuously and fed into the computer. The computer controls arena position, IR-laser (heat) activation and color of illumination according to the conditioning rules. (*Right*) Experimental schematics used in this study. Patterns and colors depict the wall of the cylinder surrounding the fly. Colored boxes indicate the four 90° quadrants. Red boxes in the example color/pattern schematics depict heated quadrants. Note that even though quadrants may be drawn in different colors here, the illumination of the entire arena is always changed. See Materials and Methods for a detailed description.

mushroom-body output. To test these hypotheses, we have designed a set of experiments in which the same stimuli are arranged to produce both context-dependent and independent pattern memory in wild-type flies (Fig. 2). To start exploring the biological basis of context dependence, we tested transgenic flies in some of these experiments (Fig. 4, below).

Results

Outline

We observed a shift in associative strength of the contextual color cues with increasing predictive relationship of the colors to the punishing heat beam (Fig. 2). The increase in the predictive relationship was conducted in five steps. The first step was to reproduce the original context generalization experiment by Liu et al. (1999) with wild-type flies and also with the mb247 driver strain used here (driving tetanus-toxin expression in the mushroom bodies). In this case, the predictive relationship was minimal, as the color change only occurred once, and this single signal predicted a test period (Fig. 2A, Context generalization). In a second step, the background color change still only predicted test from training, but this training-test transition now occurred repeatedly instead of only once (Fig. 2B,C, Feature discrimination). In a third step, background colors were still changed between periods as before (i.e., temporally), but this time the color change did not simply indicate the training-test transitions, but indicated a reversal of the CS–US contingency; one color indicated that the upright T was punished (and the inverted T unpunished), while the other color indicated the reverse relationship (Fig. 2D, Temporal conditional discrimination). In a fourth step, the colors still indicated the nature of the CS–US contingency as in the step before, but instead of only controlling the patterns while the colors were switched between periods, the flies now had operant control over both colors and patterns (Fig. 2E, Operant spatial conditional discrimination). A yoked control established the importance of this additional operant component (Fig. 2F, Yoked conditional discrimination). At the same step, we also established classical conditional discrimination (Fig. 2G, Classical spatial conditional discrimination). In a fifth and final step, we set up the background colors as direct predictors (CS) of the US (Fig. 2H, Operant color discrimination learning). Thus, in a battery of tests, we varied the predictive value of the same contextual color cues in a stepwise fashion from minimally predictive (Step 1) to indirectly predictive (Steps 2–4) to directly predictive (Step 5). Would the flies follow this scheme and shift their processing of the colors from generalization to discrimination?

To investigate the biological basis of context dependence and to provide a proof of concept for the neurobiological value of such a closed methodology, we tested flies with experimentally blocked mushroom-body output in both context generalization and two cases of conditional discrimination (Fig. 4, below).

Colors as context

At the first step, wild-type flies master the context generalization task (Fig. 2A, Context generalization), while the transgenic flies with blocked mushroom-body output fail to generalize the pattern memory across the contexts (Fig. 4A, below, Context generalization). This result corroborates and extends the results by Liu et al. (1999), which did not include the driver strain mb247. Thus, the wild-type animals did not reveal whether they had detected the context change and showed the conditioned pattern preference even in the new context. This result simultaneously corroborates previous findings (Brembs and Heisenberg 2001; Tang and Guo 2001) that flies can process patterns independently from colors and do not treat the two sets of stimuli as a compound. It needs to be pointed out that flies with impaired mushroom-body function are otherwise fairly normal and, for example, readily learn to discriminate the visual patterns operantly and classically (Wolf et al. 1998; Liu et al. 1999).

Colors in a feature discrimination task

In the context generalization experiment, the background illumination is only changed once, giving this change in the total stimulus situation minimal predictive value. In our second step, we increased the predictive value of this change by increasing the number of color changes together with the application of the reinforcer. Switching between training and test periods every minute reduces the final test score only if training and test phases are characterized by different background colors (see Fig. 2B, Feature discrimination and Fig. 2C, Constant context). The flies have learned to conditionally

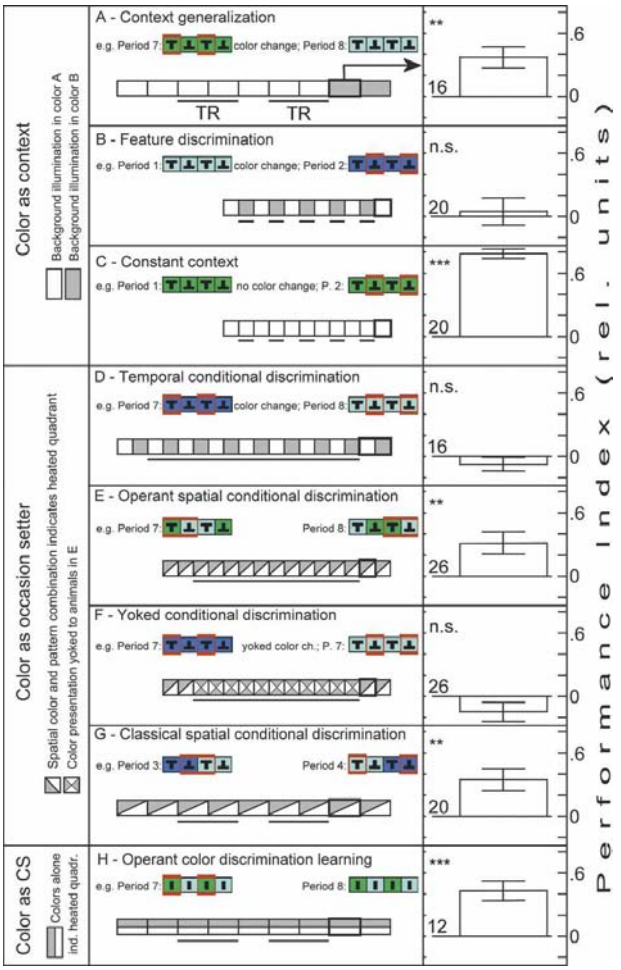


Figure 2. Colors can be context, occasion setters, and conditioned stimuli, depending on the temporal arrangement with the unconditioned stimulus. (*A,B,C*) Color as context. The color of background illumination during operant visual pattern discrimination learning changes according to the experimental schedule. (*A*) Context generalization. A single change of background illumination after the final training period marks the beginning of a 2-min test period for pattern memory in the new background color. The flies are able to show pattern memory in the new context. (*B*) The color changes are concomitant with the change from training to test periods. Increasing the number of context changes with respect to *A* abolishes the generalization effect —feature discrimination. (*C*) Control group in which training and test periods alternate in constant background color. Alternating training and test periods as in *B* does not abolish the memory score. (*D–G*) Color as occasion setter. (*D*) Color changes indicate the reversal of the pattern-heat contingency—temporal conditional discrimination. Reversal learning cannot be facilitated by context changes. (*E,F,G*) Colors change independently of the experimental schedule and indicate heated quadrants in conjunction with visual patterns. (*E*) Flies can solve a fully operant spatial conditional discrimination paradigm. (*F*) Flies fail to solve a temporal conditional discrimination task, where the color presentations are yoked to the animals in *E*. (*G*) Flies can solve spatial conditional discrimination with classical training and operant test periods. (*H*) Color as CS. The colors used in this study can be discriminated by wild-type flies in an operant visual learning task, with the colors as conditioned stimuli. Colored boxes with patterns illustrate the experimental design as in Figure 1 (see Materials and Methods for details). White/gray squares indicate 1-min. periods, rectangles 2-min.periods in the experimental time course. The performance indices of the highlighted test periods (bold) are displayed in the bar-graphs on the *right*. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; (n.s.) not significant. Numbers next to bar graphs indicate number of animals. Lines under experimental periods (indicated by "TR" in *A*) denote training periods. Performance index: $PI = (t_a-t_b)/(t_a+t_b)$.

avoid the heated patterns only in the illumination in which the patterns were actually combined with heat. Thus, the flies are able to detect that the change in coloration can predict a change in the heating regime (for more details see Wiener 2000). One could say that in this procedure, the colors that used to be part of the context are now "setting the occasion" for the punishment of certain pattern orientations, such that a pattern memory that at first was context-independent (Fig. 2A) becomes now context-dependent (Fig. 2B). In this interpretation, the paradigm is classified as feature discrimination, a case of occasion setting (Bouton and Nelson 1994; Holland et al. 1997; Dibbets et al. 2002). However, more experiments are needed to unambiguously conclude occasion setting as a mechanism for solving this feature discrimination task. We conclude that pattern memory can be both context independent and dependent, depending on the temporal relationship of the involved stimuli.

Colors in conditional discrimination tasks

Taking these experiments an additional step further, we trained the animals to avoid one set of patterns in one color (say the upright T's in green color) and the other set in another color (for instance, the inverted T's in blue-green), using the same alternating schedule. Such a paradigm is also a case of occasion setting and often referred to as conditional discrimination (Rescorla et al. 1985; Colwill et al. 1988a; Wilson and Pearce 1990). In our case, the colors change between experimental periods, rendering this experiment a case of temporal conditional discrimination. No significant learning scores were obtained, not even after 14 min of training (see Fig. 2D, Temporal conditional discrimination). Most learning effects at the *Drosophila* flight simulator become asymptotic after 8 min of training (Brembs and Heisenberg 2000). Of course, with a negative result it is impossible to rule out that the effect will appear with additional training (for discussion, see Brembs and Heisenberg 2001). We conclude that even the most amount of training used in this study was not able to reveal occasion setting when the CS was controlled operantly and the OS was presented in a temporal sequence (i.e., classically). Below we describe the results of an alternative training and testing regime of temporal conditional discrimination, which also did not yield a significant learning score (yoked conditional discrimination). Simultaneously, this experiment falsifies again the hypothesis that colors and patterns form unique percepts (compounds) in this case, one would expect a significant learning score as the paradigm corresponds to a simple discrimination.

In the previous experiment, only the patterns were under operant control, the colors were changed every minute by the computer program. Operant control of CSs facilitates learning about them (Brembs 2000; Brembs and Heisenberg 2000; Heisenberg et al. 2001). Possibly, operant control of the background colors would also lead to a facilitation of learning about their predictive value. Therefore, in a fourth step, we developed a scheme where the flies controlled both colors and patterns operantly. In this scheme, the color switches between quadrant borders such that flying toward an upright T will be punished in one color and unpunished in the other; the reverse contingency holds for the inverted T. In contrast to the previous experiment, the color changes take place at fixed points in space, which is why we termed it spatial conditional discrimination. The significant learning score indicates a successful operant spatial conditional discrimination experiment (Fig. 2E, Operant spatial conditional discrimination).

The standard experiment determining the importance of operant control is a so-called "yoked" control, where the stimuli

under investigation are delivered to the animal in the same temporal sequence in which operantly trained animals experienced them. In our version of the yoked control, the patterns were under operant control of the fly, but the colors were changed according to the sequence stored during the experiment (Fig. 2E), where both colors and patterns had been under operant control (a so-called replay experiment) (Wolf and Heisenberg 1991; Brembs and Heisenberg 2000). Whenever the color changed, the pattern-heat association was reversed according to the stored sequence of color changes by the previously trained flies. The animals could thus control their flight direction with respect to the patterns operantly, but the pattern-heat association changed with the illumination of the arena independently of their behavior (Fig. 2F, Yoked conditional discrimination). For instance, if the fly was flying toward an unpunished upright T in blue-green arena illumination, the color would change to blue (and the heat turn on) whenever the animal it was yoked to had changed arena coloration (without changing the position of the arena for the current fly). We favored this approach over a yoked control where both color and pattern sequences were played back, because it has already been shown that pattern replay does not support conditioning in the timeframe used here (Wolf and Heisenberg 1991; Brembs and Heisenberg 2000). Training with operant control of the patterns and yoked presentation of the colors did not yield significant learning scores (Fig. 2F). This result is in line with the failure to acquire a temporal conditional discrimination (Fig. 2D), as the yoked procedure also changes the color background according to a temporal regime, albeit not at regular intervals, but at the time points specified in the stored sequence. In this respect, the yoked control experiment amounts to a transfer experiment in which the flies are trained in a temporal conditional discrimination paradigm and are tested in a spatial conditional discrimination paradigm. Interestingly, the yoked control group also exhibits significantly lower heat avoidance (repeated measures ANOVA, SS: 81.44, df: 1, MS: 81.44, $F_{(278.4)}$, $P < 0.001$; data not shown). Apparently, exafferent inversion of the pattern-heat contingency is detrimental for heat avoidance, even if it is signaled by a concomitant change in background coloration. In conclusion, the yoked control experiment demonstrates that it is neither the number nor the dynamics of the color changes that lead to successful conditional discrimination when the animals control both colors and patterns operantly (Fig. 2E). Consequently, the arrangement of operant control over both patterns and colors enabled the animals to learn that color predicts the nature of the pattern-heat contingency.

Operant control facilitates conditional discrimination

We also investigated whether spatial conditional discrimination could be accomplished entirely classically, i.e., by training the animals with the same spatial arrangement of colors and patterns as in the fully operant experiment, but independently of their behavior. To this end, the arena was rotated slowly around the animal in open loop. Colors and heat were switched according to the same rules as during the fully operant experiment, i.e., between the patterns. Thus, the animals could learn about the colors predicting the nature of the pattern-heat contingency, as in the other conditional discrimination tasks, but this time the colors were arranged with the same spatial relationship to the arena position as in the fully operant experiment. *Drosophila* can, in principle, be classically conditioned to learn this occasion setting situation (Fig. 2G, Classical spatial conditional discrimination). However, the classical procedure was performed by exposing the flies to equal amounts of heat and no-heat, whereas an operant occasion setting yielded significant results with the flies avoiding the heat for about 86% of the training periods (average training PI = 0.72). Possibly, even more training would also lead to a significant learning effect in the temporal conditional discrimination task (Fig. 2D). This difference in heat requirement indicates a more efficient conditional discrimination if all predictive stimuli are under operant control. Previous work on operant and classical discrimination learning has led to the hypothesis that operant control of environmental stimuli (i.e., composite operant conditioning) facilitates the acquisition of classical (i.e., CS–US) associations (Brembs 2000; Brembs and Heisenberg 2000; Heisenberg et al. 2001). Our results seem to suggest that learning about OSs is also facilitated by operant control.

There remains only one possibile explaination of our conditional discrimination results thus far. The animals might detect which of the two color changes is associated with heat and no-heat, respectively, and then chose flight directions with respect to these references exactly between two patterns. In this view, the orientation of the patterns (i.e., upright or inverted T) would be irrelevant. We approached this possibility analytically and experimentally (Fig. 3). First, we plotted the time spent in each heated and nonheated semicircle of the arena, respectively. A fixation peak at the angular position exactly between two patterns (i.e., where the colors switch) would support the alternative explanation. However, the flies chose flight directions preferentially toward the patterns and not between two patterns (Fig. 3A). Nevertheless, the flies might only need a short period (or even only one instance) of switching from one color to the other in order to detect the safe flight directions, only to then continue to fixate the "safe" patterns. Therefore, we have conducted a control experiment identical to the operant spatial conditional discrimination paradigm (Fig. 2E) but with the patterns replaced by four identical, vertical stripes. If the flies learn to solve the operant spatial conditional discrimination paradigm by a simple association between turning direction, color switch, and heat, the pattern orientations should be irrelevant and the flies perform equally well in this control procedure. However, the flies do not show a significant learning score, falsifying this hypothesis and lending additional support to our hypothesis that indeed the logical combination between pattern orientations and color is learned in our case of occasion setting (Fig. 3B).

Colors as conditioned stimuli

Finally, in the fifth step, we conducted an operant color-discrimination learning experiment similar to the one developed earlier (Wolf and Heisenberg, 1997). In this experiment, the same colors that were used as context and OS in the experiments described above were set up to predict the occurrence of each single heating episode (i.e., the colors were set up as CS). In order to successfully solve this learning task, the fly has to choose "safe" flight directions with respect to four identical stripes. The only predictor of punishment is the arena coloration that switches between the identical stripes according to the conditioning schedule. *Drosophila* can learn to associate one of the colors with heat and avoid flight directions that lead to an arena illumination of this color (Fig. 2H, Operant color discrimination learning). A priori, this was not necessarily to be expected, considering that the colors used by Wolf and Heisenberg (1997), in contrast to the colors used here, do not support context generalization when used as context (Liu et al. 1999). However, in light of the successful conditional discrimination experiments, the suitability of these colors as CSs comes as somewhat less of a surprise. This result also emphasizes that the flies can readily distinguish the colors, even though they do not reveal that they can detect the color change in the wild-type context generalization experiment.

Summarizing the wild-type results, it emerges that the color pairs used for successful context generalization (i.e., blue-green/blue and blue-green/green) are also suitable to serve both as OS

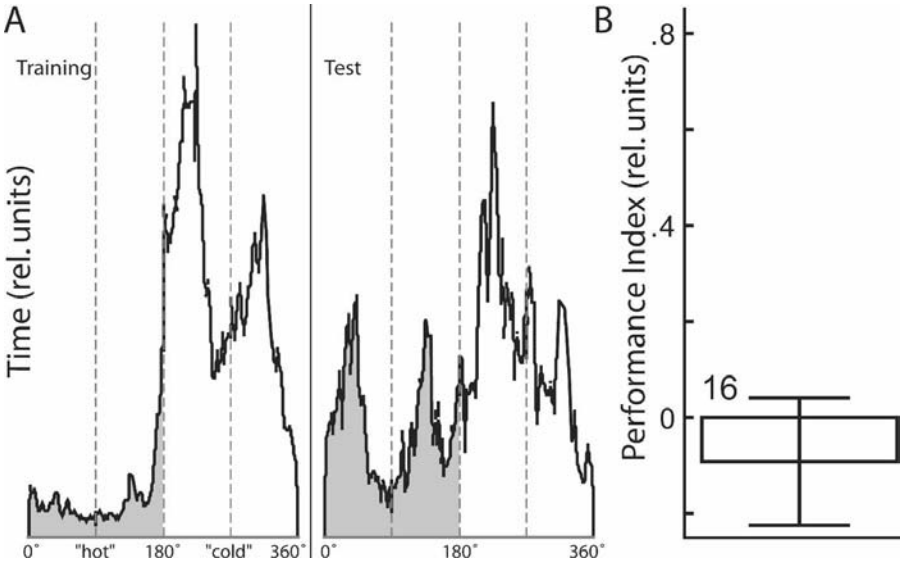


Figure 3. Operant conditional discrimination learning is not due to simple conditioning. (*A*) Summed fixation histograms of the last 7 min for all 26 flies in the operant conditional discrimination experiment depicted in Figure 2E. (*Left*) Relative time spent at flight directions of 0–360° with respect to one of the hot/cold borders during the last 5 min of training. (*Right*) Flight directions during the following two minutes of test. The flies fixate the patterns preferentially both during training and during test. (Shaded area) Quadrants associated with heat; (white area) quadrants associated with heat-off; (dashed lines) quadrant borders between T-patterns. (*B*) Performance index of a control experiment in which patterns and colors were arranged as in an operant spatial conditional discrimination experiment (Fig. 2E), but with the T-shaped patterns replaced by four identical stripes as in color discrimination learning (Fig. 2H). No significant performance index was obtained. Performance index: $PI = (t_a\text{-}t_b)/(t_a+t_b)$.

and as CS. It can be concluded that a contextual stimulus can in principle become OS or CS, depending only on its temporal relationship to the US. In this light, both context dependence and independence appear as features of the brain.

Mushroom bodies are required for context generalization, but are dispensable for conditional discrimination

Knowing that flies without functional mushroom bodies fail to generalize between contexts (Fig. 4A, Context generalization; Liu et al. 1999), but do learn colors and patterns as CSs (Wolf et al. 1998), we tested transgenic flies with blocked mushroom-body output for their ability to perform conditional discrimination (Fig. 4B,C). Possibly, flies without functional mushroom bodies only fail to generalize between contexts because they learn quicker than wild-type flies that there will not be any punishment in the new context (see Figs. 2A, 4A; in other words, flies without mushroom bodies may exhibit facilitated conditional discrimination). The unsuccessful temporal conditional discrimination experiment for these flies (Fig. 4B, Temporal conditional discrimination) falsifies this hypothesis, as one would have expected the facilitated learning to improve performance also in this task over wild type. The negative results from both wild-type (Fig. 2D) and transgenic flies (Fig. 4B) in this experiment also corroborate the findings (this study, as well as data from Liu et al. 1999; Brembs and Heisenberg 2001; Tang and Guo 2001) that colors and patterns are processed separately and not as compounds (even in flies with impaired mushroom-body function!).

Interestingly, flies with blocked mushroom-body output did produce significant performance indices after training in our fully operant, spatial version of the occasion setting paradigm (Fig. 4C, Operant spatial conditional discrimination).

Discussion

Higher-order learning in *Drosophila*

For a learning situation to be classified as occasion setting, three criteria have to be met (Young et al. 2000; Pearce and Bouton 2001; Law et al. 2004). First, the two stimuli have to be processed individually and not treated as a compound. Second, the OS does not enter into an association with the US alone (no simple conditioning of the OS). Third, the OS has to be a specific modulator of the CS. We have corroborated previous evidence for the separate processing of colors and patterns (Brembs and Heisenberg 2001) by showing that our choice of colors and patterns are indeed separable (Fig. 2A). Indeed, we have shown that the animals have to learn to incorporate the colors into the pattern memory template (Fig. 2B). Additionally, one of our conditional discrimination paradigms (Fig. 2D, Temporal conditional discrimination) should have shown a significant learning score if colors and patterns had been processed as compounds. Moreover, even different pattern memories are processed by different layers of the fan-shaped body (Liu et al. 2006), making innate compound processing of pattern and color memories unlikely. Thus, there are several independent lines of evidence suggesting that the first criterion is met. In vertebrates, extinction or transfer experiments have conventionally been used to meet the second criterion (Bouton and Swartzentruber 1986; Holland 1986, 1989a,b; Myers and Gluck 1994; Young et al. 2000; Pearce and Bouton 2001; Law et al. 2004). These studies typically involved simple Pavlovian conditioning procedures with a single OS indicating the presence or absence of the US (i.e., feature discrimination, see, e.g., Bouton and Nelson 1994; Holland et al. 1997; Young et al. 2000; Pearce and Bouton 2001; Dibbets et al. 2002). These feature discrimination designs (e.g., Fig. 2B) do indeed require such additional controls, as the OS may be associated with the US alone. However, our final conditional discrimination designs are fully symmetrical (Fig. 2D,E,G), i.e., both OS and CS come in an equally nonpredictive pair. Both the color pair and the pattern pair are by themselves equally associated with heat and therefore unsuitable as predictors of the heat—50% of both colors are associated with heat and 50% of both pattern orientations. Consequentially, neither of the two stimuli can enter into the association alone (simple conditioning cannot take place). Only by using the logical combination between the two (reminiscent of a configural learning task) (see, e.g., Pearce 1987, 1994; Young et al. 2000; Pearce and Bouton 2001), can the fly solve our conditional discrimination task (thus meeting the second criterion). Finally, our conditional discrimination designs use all possible permutations of colors and pattern orientations, excluding the possibility of the colors serving as a general (unspecific) modulator on the patterns—they must be learned as specific modulators of the pattern-heat contingency and thereby meet the third criterion.

We interpret these results as evidence that the flies solve the conditional discrimination tasks via an occasion-setting mechanism. To our knowledge, such higher-order learning has been found in only a few invertebrates (Colwill et al. 1988a; Rogers et

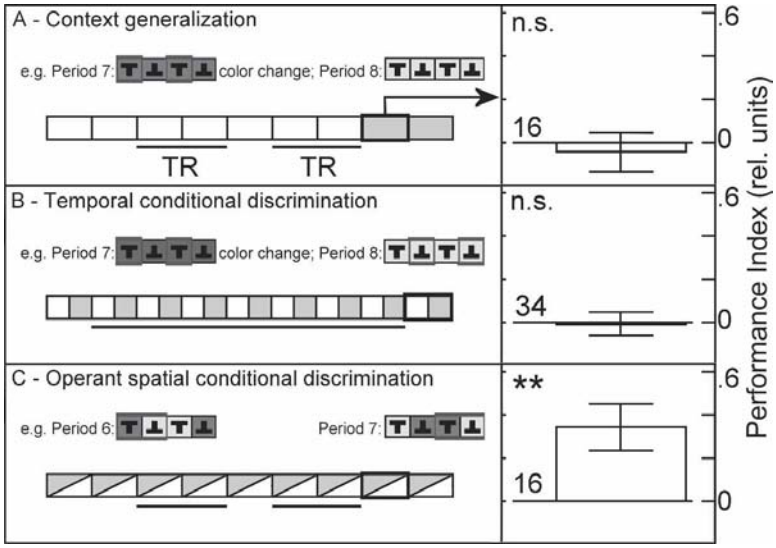


Figure 4. Flies with blocked mushroom-body output fail in context generalization and temporal conditional discrimination, but perform in spatial conditional discrimination. (*A*) Context generalization as in Figure 2A. Flies with blocked mushroom-body output do not transfer the pattern memory acquired during training to a different background color. (*B*) Temporal conditional discrimination where the colors in each period indicate the nature of the pattern/heat contingency (as in Fig. 2D). As in wild-type flies, color changes do not facilitate reversal learning and, hence, are not learned as occasion setters in flies with blocked mushroom-body output. (*C*) Operant spatial conditional discrimination as in Figure 2E, but with altered period set-up and duration. Colored boxes with patterns illustrate the experimental design as in Figure 1 (see Materials and Methods for details). White/gray rectangles indicate 2-min. periods, as in Figure 2. Performance indices of highlighted (bold) test periods are displayed in the bar-graphs on the *right*. $^{**}P < 0.01$; (n.s.) not significant. Numbers next to bar graphs indicate number of animals. Lines under experimental periods (indicated by "TR" in *A*) denote training periods. Performance index: $PI = (t_a\text{-}t_b)/(t_a+t_b)$.

al. 1996; Schubert et al. 2002; Law et al. 2004; Matsumoto and Mizunami 2004) and has never been shown to be facilitated by operant control of the involved stimuli. This opens up higher-order conditioning for investigation in a genetically tractable, small-brained model system. Interestingly, free-flying honey bees are able to solve an operant conditional discrimination task (Schubert et al. 2002), whereas such a paradigm has proven more difficult to develop and is still lacking in the classical proboscis extension reflex with harnessed bees.

Mushroom bodies stabilize memory templates during context changes

Our approach opens the exciting aspect to investigate the neural mechanisms of higher-order associative processing. In simple associative conditioning, temporal coincidence is sufficient for individual neurons to mediate the learning process (Carew et al. 1981; Kandel and Schwartz 1982; Walters and Byrne 1983; Murphy and Glanzman 1999; Brembs et al. 2002; Antonov et al. 2003). In occasion setting, each stimulus by itself will trigger coincident firing where its pathway and the one of the US converge, since each separately will be reinforced—only the logical combination determines an unambiguous rule. Hence, the biological modifications in the brain need to take place in brain regions where the logical connection between OS and CS is preserved and not where the CS or the OS themselves are processed. Interestingly, memory traces for visual patterns have been localized to different layers of the fan-shaped body (Liu et al. 2006), while the brain regions implicated in the processing of CS and context are the mushroom bodies (Liu et al. 1999). Among a variety of measures that compromise mushroom-body function, we chose to use transgenic expression of tetanus toxin in mushroom-body Kenyon cells to prevent synaptic transmission. Flies with compromised mushroom-body function perform well in a range of behaviors. They show coordinated walking, the full male courtship sequence, visual flight control, and basic responses to various stimuli (Heisenberg et al. 1985; Heisenberg and Wolf 1988; de-Belle and Heisenberg 1994; Connolly et al. 1996). While they can solve a number of learning tasks (Wolf et al. 1998), they fail in context generalization (Liu et al. 1999). In a first demonstration of the powerful combination of a closed behavioral methodology and modern transgenics, we have subjected flies with blocked mushroom-body output to both context generalization and occasion-setting experiments. While the flies failed to express pattern memory after a context change (Fig. 4A) (extending previous results from Liu et al. 1999), they could use the predictive information in the context change to learn about the pattern-heat contingency and solve the operant conditional discrimination task (Fig. 4C). Although we consider it unlikely, it would nevertheless be interesting to test more mushroom-body-specific driver lines to possibly find strains that fail in both tasks. Additionally, converging results from redundant techniques such as the ablation of the mushroom bodies by treating larvae with hydroxy-urea (de-Belle and Heisenberg 1994) would serve to corroborate any findings in other transgenic lines. The mushroom bodies are a prominent neuropil and a hotspot of research (Heisenberg 2003; Gerber et al. 2004). Among many hypotheses, it has been proposed that mushroom bodies reduce the sensitivity to context changes by first extracting the CS from the context and then stabilizing the CS–US memory template against context changes (Liu et al. 1999). One original hypothesis was that flies without functional mushroom bodies cannot extract the CS from the context as well as wild-type flies and, hence, are not able to express the memory in the new context (Liu et al. 1999). An alternative hypothesis explains the context dependence in mushroom-body-impaired flies with enhanced occasion setting. Our temporal conditional discrimination task can discriminate between these alternatives. If mushroom bodies are involved in the separation of CS from context, mushroom-body-impaired flies should at least show a small learning score after 14 min of training (Fig. 4B), as they only have to solve two simple conditioning tasks instead of a higher-order task. If mushroom bodies reduce the capacity to learn occasion setting, mushroom-body-impaired flies should show a significant learning score already after <14 min of training. However, the mushroom-body-impaired flies completely failed this task, just as the wild-type flies. Suppose our results also hold for other transgenic lines as well as other, redundant techniques, what would this mean for our understanding of mushroom-body function?

Our data show that flies with impaired mushroom-body output probably can extract the patterns from the color background (Fig. 4B). If the colors were just part of the memory template, as Liu et al. (1999) suggest, the temporal conditional discrimination paradigm should amount to two simple conditioning tasks, e.g., the upright T in blue background is something

entirely different from the upright T in blue-green background. Flies with impaired mushroom-body function can solve such simple conditioning tasks (Wolf et al. 1998) and there should be no interference between the two—flies can store at least four memory templates simultaneously (Heisenberg et al. 2001). Thus, there appears no reason why these flies should not, in principle, be able to solve the task if patterns and colors were indeed not separable for them. It is tempting to even go so far as to interpret this result as evidence for context-independent memory in mushroom-body-impaired flies, but further experiments are needed to corroborate this interpretation.

On the same grounds, the data also rule out the alternative hypothesis that flies with impaired mushroom-body function are more efficient in occasion-setting tasks. If the reduction in avoidance after a context change (Fig. 4A) were indeed due to occasion setting, the temporal conditional discrimination task (Fig. 4B) should show a significant learning score already early in the experiment.

In conclusion, flies without mushroom bodies still appear to separate colors and patterns, but do not show enhanced occasion setting. With such evidence against both hypotheses, the picture instead emerges that mushroom bodies may specifically enhance the stability of memory traces against changes in the stimulus situation, but do not decrease the ability to detect such changes. Higher-order learning (occasion setting) appears to be independent of this function of the mushroom bodies. There is evidence suggesting that the reason for this independence lies in the different processing of generalization vs. discrimination tasks (Brembs and Hempel de Ibarra 2006).

Context dependence can be both feature and failure

One can intuitively understand the value both in heeding and in ignoring a signal for a biologically important event in a new situation. The former recognizes that the signal may still be relevant, even in the new situation; the latter saves valuable resources by recognizing that in the new situation the signal needs to validate its signaling qualities. One can also easily understand that the degree to which the two situations differ has an effect on the outcome of such experiments. Indeed, the choice of colors is important for context-independent or context-dependent pattern memory in *Drosophila* (Liu et al. 1999). Presumably, and this is precisely where the issue becomes more intricate, the crucial determinant of context dependence or independence are the (species-specific) stimuli that make up what the human observers call "context." For some researchers, context generalization is the brain capacity and biological manipulations are sought to compromise it (e.g., Liu et al. 1999). For others, the context dependence is the brain capacity (occasion setting) and biological manipulations are sought to compromise it (e.g., Law et al. 2004). The conundrum is not enlightened by a large number of terms denoting the same or very similar experimental situations. Context generalization (this study; Liu et al. 1999; Brembs and Hempel de Ibarra 2006) takes place when a memory can be transferred between different contexts (context-independent memory). Contextual learning or context conditioning (e.g., Colwill et al. 1988b; Kim and Fanselow 1992; Rogers and Matzel 1996; Debiec et al. 2002) refers to experiments in which different contexts predict the occurrence or nonoccurrence of otherwise unsignaled USs. Feature discrimination (e.g., Bouton and Nelson 1994; Holland et al. 1997; Dibbets et al. 2002) refers to experiments where a stimulus (or context) predicts the reinforcement/nonreinforcement of a CS. Conditional discrimination (e.g., Rescorla et al. 1985; Colwill et al. 1988a; Wilson and Pearce 1990), trans-switching (e.g., Furedy 1991; Lachnit and Kimmel 1991), and ambiguous discrimination (e.g., Holland 1991) all denote experiments that are also classified as occasion setting (e.g., Swartzentruber 1991; Bonardi and Hall 1994; Miller and Oberling 1998; Schmajuk et al. 1998; Young et al. 2000; Clarke et al. 2001)—a stimulus (or context) characterizes the nature of a CS–US contingency or discrimination. Traditionally, researchers distinguished between predictive stimuli and mere "context" either by the physical properties of the stimuli (e.g., Bouton et al. 1999), or according to their temporal relationship to the US (e.g., Wickens 1987). The work on *Drosophila* at the flight simulator demonstrated that both physical properties (Liu et al. 1999; Brembs and Hempel de Ibarra 2006) and the nature of the predictive relation to the reinforcer (this study) are critical for the decision of whether to treat two situations as equivalent or as fundamentally different. The fewer the changes between situations, the more pronounced the impact of the physical properties of the situation (Liu et al. 1999; Brembs and Hempel de Ibarra 2006); the more changes, the more pronounced the role of the changes and their relationship to the reinforcement (this work; see also Swartzentruber 1991; Myers and Gluck 1994). Thus, for the general organization of learning experiments, it must be emphasized that the classification of stimuli as "context" is less obvious and self-explanatory than it might seem. Moreover, whether the nonretrieval of a memory in any however slightly altered experimental situation can be considered a feature (contextual memory) or a failure (no generalization) cannot be addressed without further experiments of the kind detailed in this work. The overarching brain capacity is to be able to flexibly generalize or discriminate between two situations depending on the information the difference between the situations conveys to the animal. Investigating generalization or discrimination individually is a one-sided endeavor and may thus yield confusing results. In our companion paper, we have applied this new methodology and addressed the inter-dependence of the physical parameters and the predictive value to show that discrimination and generalization of background colors are supported by different parameters in *Drosophila* (Brembs and Hempel de Ibarra 2006).

For fruit flies, as for humans, the claim appears valid that "like parallel research on occasion setting, research on contextual control suggests that a more complex associative structure may often be acquired in associative learning." (Pearce and Bouton 2001).

Materials and Methods

Flies

Flies are kept on standard cornmeal/molasses medium (Guo et al. 1996) at 25°C and 60% humidity with a 14-h light/10-h dark regime. Females aged 24–48 h are briefly immobilized by cold-anesthesia and glued (Loctite UV glass glue) with head and thorax to a triangle-shaped copper hook (diameter 0.05 mm) the day before the experiment. The animals are then kept individually overnight in small moist chambers containing a few grains of sucrose.

Transgenes

Sweeney et al. (1995) developed a method that constitutively blocks synaptic transmission by expressing the catalytic subunit of bacterial tetanus toxin (Cnt-E) in target neurons in the *Drosophila* brain using the P[GAL4] technique (Brand and Perrimon 1993). Because of the effects of mushroom-body function on context generalization (Liu et al. 1999), we use the Cnt-E transgene to block synaptic output from the mushroom bodies. The P[GAL4] line mb247 (Schulz et al. 1996) is used as a mushroom-body-specific GAL4 driver (Zars et al. 2000). This driver strain has not been tested for context generalization previously. We use the trans-heterozygote offspring from the driver (mb247) and the

reporter strain (UAS_{GAL4}-Cnt-E) for our studies as described previously (Sweeney et al. 1995; Baier et al. 2002).

Apparatus

The *Drosophila* flight simulator is a computer-controlled feedback system in which the fly uses its yaw torque to control the rotations of a panorama surrounding it (Fig. 1). The core device is the torque meter (Götz 1964; Heisenberg and Wolf 1984), which measures a fly's angular momentum around its vertical body axis. The fly, glued to the hook, is attached to the torque meter via a clamp to accomplish stationary flight in the center of a cylindrical panorama (arena; diameter 58 mm), homogeneously illuminated from behind (Fig. 1). The light source is a 100W, 12V tungsten-iodine bulb. For background coloration of the arena, the light is passed through one of three different broad band filters—(1) broadband blue (Kodak Wratten gelatin filter No. 47); (2) broadband green (Kodak Wratten gelatin filter No. 99); and (3) "daylight" blue-green (Rosco "surfblue" No. 5433). The transmission spectrum of the Rosco blue-green filter used in this study is equivalent to that of the BG18 filter (Schott, Mainz) used by Liu et al. (1999) (data not shown). Filters can be exchanged by a fast solenoid within 0.1 sec.

A computer-controlled electric motor rotates the arena such that its angular velocity is proportional to, but directed against the fly's yaw torque (coupling factor $K = -11°/sec \cdot 10^{-10}$ Nm). This enables the fly to stabilize the panorama and to control its angular orientation. This virtual "flight direction" (i.e., arena position) is recorded continuously via a circular potentiometer (Novotechnik, A4102a306). An analog to digital converter card (PCL812; Advantech Co.) feeds arena position and yaw torque into a computer that stores the traces (sampling frequency 20 Hz) for later analysis.

Punishment is achieved by applying heat from an adjustable infrared laser (825 nm, 150 mW), directed from behind and above onto the fly's head and thorax. The laser beam is pulsed (~200 msec pulse width at ~4 Hz) and its intensity reduced to assure the survival of the fly.

General experimental design

Each fly is used only once. The time-course of the experiment is divided into consecutive periods of either 1 or 2 min duration. Depending on whether heat may be applied during such a period, it is termed a training period (heating possible) or a test period (heat off). Note that this nomenclature is independent of any higher-order training that may encompass several training/test periods. The treatment of the flies during these periods determines the type of experiment, as described below. Color pairs were always green/blue-green and blue/blue-green (Fig. 1).

Discrimination learning—patterns

For patterns as CS (Wolf and Heisenberg 1991), four black, T-shaped patterns of alternating orientation (i.e., two upright and two inverted) are evenly spaced on the arena wall (pattern width $\psi = 40°$, height $\theta = 40°$, width of bars = 14°, as seen from the position of the fly). A computer program divides the 360° of the arena into four virtual 90° quadrants, the centers of which are denoted by the patterns. During training periods, heat punishment is made contiguous with the appearance of one of the pattern orientations in the frontal visual field. Reinforcement of each pattern is always equalized within groups. During test periods, the heat is permanently switched off.

Discrimination learning—colors

For colors as CS (Wolf and Heisenberg 1997) the centers of the four virtual quadrants are denoted by four identical vertical stripes (width $\psi = 14°$, height $\theta = 40°$). The color of the illumination of the whole arena is changed whenever one of the virtual quadrant borders passes a point in front of the fly. During training periods, heat punishment is made contiguous with one color of the pairs blue/blue-green and green/blue-green. Reinforcement of each color is always equalized within groups. During test periods, the heat is permanently switched off. See Figures 1 and 2H, Operant color discrimination learning.

Context generalization

Testing for the stability of pattern memory, the number of background color changes with each training/test period is varied.

1. Following the original context generalization experiment by Liu et al. (1999), only one color change takes place after seven 2-min periods (2 × test, 2 × training, test, 2 × training), before the final 2-min test period. The color is changed either between green and blue-green for half of the cases or between blue and blue-green for the rest of the cases, such that each color is training or test color in 25% of all experiments. A successful generalization experiment is characterized by a positive learning score, which indicates that the pattern memory was generalized across the different color contexts. Such a successful experiment also shows that the pattern can be processed independently from the color and the two stimuli are not perceived as a compound (Brembs and Heisenberg 2001). Context generalization is different from context conditioning where the animals learn to respond to a context. In this study, we never performed context conditioning, but only tested for the ability of a context change to disrupt the transfer of operant pattern memory between contexts. Successful context generalization is characterized by a lack of response to the context change. See Figures 1, 2A, and 3, Context generalization.
2. In a modification of the context generalization experiment, the number of context changes is increased from one (before the final test period; see above) to 10 (between every training and test period; see below). Specifically, the duration of experimental periods is reduced to 1 min and training and test periods alternated. In this manner, five training and five test phases alternate after 1 min pre-test in the test color. Such a design increases the predictive value of the colors, as they indicate whether flying toward the pattern is punished or not. That is, if the arena is illuminated with one color, flying toward one of the patterns is punished; if illuminated with the other color, none of the patterns is punished. The use of the pattern pairs (blue/blue-green and green/blue-green) is balanced between animals. After training in this manner, the animals are subsequently tested for pattern preference in both colors, with the heat permanently switched off. The sequence of training and test coloration is balanced between animals. See Figures 1 and 2B, Context indicates heat on/off. In a control procedure, animals were subjected to pattern discrimination learning with stationary colors (Figures 1 and 2C, Constant context).

Occasion setting

In a modification of the previous experiment, arena coloration is used to indicate the nature of the pattern-heat contingency. For instance, flying toward the upright T is punished under green illumination and the inverted T is unpunished, but then blue-green illumination indicates the reverse pattern-heat contingency. In this experiment, neither of the stimuli alone can unambiguously predict reinforcement. The animals can master this discrimination only by considering the different combinations of the stimuli. We use four different occasion setting paradigms as described in the following paragraphs.

1. Temporal conditional discrimination

This version is the direct extension of the context generalization experiments described above. By alternating 1-min periods, the patterns remain under operant control. After two test periods with each arena illumination color, 14 periods of training follow. The arena illumination color changes with each period. Pattern-heat contingencies alternate with the colors (colors balanced) and periods. In this manner, the patterns stay under operant control of the fly for the duration of the experiment, but the colors are under the control of the experimenter and therefore

presented classically, i.e., independently of the behavior of the animal. In the final two 1-min periods, the animals are tested for their pattern preference in the two colors. The PIs of the two final test periods are averaged and tested against zero. See Figures 1, 2D, and 3B, Temporal conditional discrimination.

2. Operant spatial conditional discrimination

In this paradigm the colors do not change between periods (temporally) but between quadrants (spatially). In this way, both colors and patterns come under operant control. The center of each quadrant is still denoted by the patterns (alternating upright and inverted Ts). The difference to temporal conditional discrimination consists of the arrangement of color and heat with the quadrants. While heat was associated with two opposite quadrants (e.g., the ones with the upright T in the center) before, heat is now associated with adjacent quadrants, i.e., one with an upright and one with an inverted T. Thus, instead of being switched on or off at each of the four quadrant borders, the heat is now switched on or off at only two opposite borders. The color of the arena illumination is changed at the remaining two quadrant borders, where the heat is not switched on or off. Thus, heat is applied in two quadrants, which include an upright and an inverted T as well as the quadrant border where the background coloration is changed. Conversely, arena coloration is changed exactly between the two punished patterns and between the two unpunished patterns. In such a way, the flies get heated when they fly toward, say, a green upright T and a blue-green inverted T and switch the heat off by flying into one of the other two quadrants with a green inverted T and a blue-green upright T. One arrangement of quadrants may thus look as follows: The first quadrant features the upright T and whenever the fly enters this quadrant, the whole arena turns to blue illumination. The second quadrant features the inverted T and the arena illumination remains blue. If the fly enters the third quadrant with the upright T, the whole arena turns to blue-green. In the fourth quadrant, the inverted T is in the center, but the arena illumination stays blue-green. The heat regime is such that neither pattern nor color alone could predict reinforcement. For example, heat is switched on whenever the fly enters quadrants 2 or 3, but no heat is presented when entering quadrants 1 or 4. This heat regime is used for half of the animals, whereas the other half of the animals is not punished in quadrants 2 or 3, but quadrants 1 or 4 are punished.

The training phase lasts 11 min, and is divided into 1-min periods. After each period, the arena is set to a random position to minimize conditioning to spurious spatial cues. The spatial arrangement of patterns and colors was randomized across periods (i.e., if the patterns in quadrant 1 and 2 were "blue" and the patterns in quadrant 3 and 4 "blue-green" in one period, this association was reversed in a random selection of other periods). This randomization minimized the spatial contingency and emphasized the logical contingency between patterns, heat, and colors. After 11 min of training, the animals are tested for 1 min for their quadrant preference with the heat permanently switched off. See Figures 1 and 2E, Operant spatial conditional discrimination. Transgenic flies (Fig. 4B, Operant spatial conditional discrimination) were trained for 8 min (four 2-min training periods) and tested for 2 min in the same temporal order as described for context generalization (Liu et al. 1999), "Classical spatial conditional discrimination" (see below) and color discrimination learning (see above). In a control experiment (Fig. 3B), the four T-patterns were replaced by four identical, vertical stripes as in color discrimination learning (see above).

3. Yoked conditional discrimination

This experiment aims to test for the requirement of operant control of the colors. The animals control the position of the patterns, but the sequence of color changes is played back from the animals previously trained in the previous experiment (patterns and colors operant), where they controlled both the patterns and the colors operantly. Thus, the dynamics and frequency of color changes are identical to the one where arena coloration is controlled by the fly. Only in this experiment, the color change is independent of the animal's behavior. Every change in the background coloration reverses the pattern-heat contingency as before. This experiment is almost identical to the temporal conditional discrimination experiment above, with the exception of the temporal sequence of color changes matching those of spatial conditional discrimination and the test being identical to that of spatial conditional discrimination. After 11 min of training, the animals are tested for 1 min for their pattern-color preference with patterns and colors under operant control, as in the previous experiment (patterns and colors operant). See Figures 1 and 2F, Yoked conditional discrimination.

4. Classical spatial conditional discrimination

This experiment is similar to the one where patterns and colors are under operant control (patterns and colors operant). However, the arena is not under the fly's control, but is instead rotated at 30°/sec, such that the full 360° of patterns, accompanying color changes, and heat are experienced by the fly, but independently of the fly's behavior. By these means it has previously been shown that flies cannot only learn to discriminate patterns (Brembs and Heisenberg 2000) but also colors (data not shown) independently of their behavior (i.e., classically). Thus, this experiment differs from the temporal conditional discrimination in both the number of color changes as well as the spatial relationship of the color change and the arena position. It has been shown previously that flies are sensitive to such spatial information (Brembs and Heisenberg 2000). As in the fully operant experiment, the spatial arrangement of patterns and colors was randomized across periods (i.e., if the patterns in quadrant 1 and 2 were "blue" and the patterns in quadrant 3 and 4 "blue-green" in one period, this association was reversed in a random selection of other periods). Again, this randomization is intended to minimize the spatial contingency and emphasize the logical contingency between patterns, heat, and colors. After 8 min of training, the animals are tested for 2 min for their quadrant preference with the heat permanently switched off. In this test, both colors and patterns are under full operant control, as in the previous two experiments. See Figures 1 and 2G, Classical spatial conditional discrimination.

Data evaluation and statistics

The color and/or pattern preference of individual flies is calculated as the performance index $PI = (t_a\text{-}t_b)/(t_a+t_b)$. During training periods, t_b indicates the time the fly is exposed to the heat and t_a the time without heat. During test periods, t_a and t_b refer to the times when the fly choose the formerly (or subsequently) unpunished or punished situation, respectively. Thus, a PI of 1 means the fly spent the entire period in the quadrants not associated with heat, whereas a PI of -1 indicates that the fly spent the entire period in the quadrants associated with heat. Accordingly, a PI of zero indicates that the fly distributed the time evenly between heated and nonheated quadrants. PIs from test periods are called "test PIs" or "learning scores." Learning scores were tested for significance using a *t*-test for single means against zero, following Liu et al. (1999).

Acknowledgments

Our special gratitude goes to Bertram Gerber for vital intellectual input not only into the development of the spatial conditional discrimination paradigm, but also into the writing of this manuscript. We are indebted to Gérard Leboulle, Syed Abid Hussaini, and our Monday journal club for commenting on an earlier version of the manuscript.

References

Antonov, I., Antonova, I., Kandel, E.R., and Hawkins, R.D. 2003. Activity-dependent presynaptic facilitation and hebbian ltp are both required and interact during classical conditioning in *Aplysia*. *Neuron* **37:** 135–147.

Baier, A., Wittek, B., and Brembs, B. 2002. *Drosophila* as a new model organism for the neurobiology of aggression? *J. Exp. Biol.*

205: 1233–1240.
Bonardi, C. and Hall, G. 1994. Occasion-setting training renders stimuli more similar: Acquired equivalence between the targets of feature-positive discriminations. *Q. J. Exp. Psychol. B* **47:** 63–81.
Bouton, M.E. and Nelson, J.B. 1994. Context-specificity of target versus feature inhibition in a feature-negative discrimination. *J. Exp. Psychol. Anim. Behav. Process.* **20:** 51–65.
Bouton, M.E. and Swartzentruber, D. 1986. Analysis of the associative and occasion-setting properties of contexts participating in a Pavlovian discrimination. *J. Exp. Psychol. Anim. Behav. Process.* **12:** 333–350.
Bouton, M.E., Nelson, J.B., and Rosas, J.M. 1999. Stimulus generalization, context change, and forgetting. *Psychol. Bull.* **125:** 171–186.
Brand, A.H. and Perrimon, N. 1993. Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. *Development* **118:** 401–415.
Brembs, B. 2000. An analysis of associative conditioning in *Drosophila* at the flight simulator. Ph.D. thesis, University of Würzburg, Würzburg, Germany.
Brembs, B. and Heisenberg, M. 2000. The operant and the classical in conditioned orientation in *Drosophila melanogaster* at the flight simulator. *Learn. Mem.* **7:** 104–115.
———. 2001. Conditioning with compound stimuli in *Drosophila melanogaster* in the flight simulator. *J. Exp. Biol.* **204:** 2849–2859.
Brembs, B. and Hempel de Ibarra, N. 2006. Different parameters support generalization and discrimination learning in *Drosophila* at the flight simulator. *Learn. Mem.* (this issue).
Brembs, B., Lorenzetti, F.D., Reyes, F.D., Baxter, D.A., and Byrne, J.H. 2002. Operant reward learning in *Aplysia*: Neuronal correlates and mechanisms. *Science* **296:** 1706–1709.
Carew, T.J., Walters, E.T., and Kandel, E.R. 1981. Classical conditioning in a simple withdrawal reflex in *Aplysia californica*. *J. Neurosci.* **1:** 1426–1437.
Clarke, H.A., Skinner, D.M., and van der Kooy, D. 2001. Combined hippocampal and amygdala lesions block learning of a response-independent form of occasion setting. *Behav. Neurosci.* **115:** 341–357.
Colwill, R.M., Absher, R.A., and Roberts, M.L. 1988a. Conditional discrimination learning in *Aplysia californica*. *J. Neurosci.* **8:** 4440–4444.
———. 1988b. Context-us learning in *Aplysia californica*. *J. Neurosci.* **8:** 4434–4439.
Connolly, J.B., Roberts, I.J., Armstrong, J.D., Kaiser, K., Forte, M., Tully, T., and O'Kane, C.J. 1996. Associative learning disrupted by impaired gs signaling in *Drosophila* mushroom bodies. *Science* **274:** 2104–2107.
de-Belle, J.S. and Heisenberg, M. 1994. Associative odor learning in *Drosophila* abolished by chemical ablation of mushroom bodies. *Science* **263:** 692–695.
Debiec, J., LeDoux, J.E., and Nader, K. 2002. Cellular and systems reconsolidation in the hippocampus. *Neuron* **36:** 527–538.
Dibbets, P., Maes, J.H., and Vossen, J.M. 2002. Serial and simultaneous feature positive discriminations in a human skinner box. *Behav. Processes* **59:** 1–14.
Dickinson, A. and Balleine, B.W. 2002. The role of learning in the operation of motivational systems. In: *Learning, motivation and emotion, volume 3 of Steven's handbook of experimental psychology*, 3rd ed., (ed. C.R. Gallistel), pp. 497–533. John Wiley & Sons, New York.
Ernst, R. and Heisenberg, M. 1999. The memory template in *Drosophila* pattern vision at the flight simulator. *Vision Res.* **39:** 3920–3933.
Furedy, J.J. 1991. On testing current cognitive theories of Pavlovian conditioning in the human Pavlovian autonomic transswitching preparation. *Biol. Psychol.* **32:** 201–204.
Gerber, B., Tanimoto, H., and Heisenberg, M. 2004. An engram found? Evaluating the evidence from fruit flies. *Curr. Opin. Neurobiol.* **14:** 737–744.
Götz, K.G. 1964. Optomotorische untersuchung des visuellen systems einiger augenmutanten der fruchtfliege *Drosophila*. *Kybernetik* **2:** 77–92.
Guo, A., Liu, L., Xia, S.-Z., Feng, C.-H., Wolf, R., and Heisenberg, M. 1996. Conditioned visual flight orientation in *Drosophila*; dependence on age, practice and diet. *Learn. Mem.* **3:** 49–59.
Heisenberg, M. 2003. Mushroom body memoir: From maps to models. *Nat. Rev. Neurosci.* **4:** 266–275.
Heisenberg, M. and Wolf, R. 1984. *Vision in* Drosophila. *Genetics of microbehavior*. Springer, Berlin, Heidelberg, New York, Tokyo.
———. 1988. Reafferent control of optomotor yaw torque in *Drosophila melanogaster*. *J. Comp. Physiol. [A]* **163:** 373–388.
Heisenberg, M., Borst, A., Wagner, S., and Byers, D. 1985. *Drosophila* mushroom body mutants are deficient in olfactory learning. *J. Neurogenet.* **2:** 1–30.
Heisenberg, M., Wolf, R., and Brembs, B. 2001. Flexibility in a single behavioral variable of *Drosophila*. *Learn. Mem.* **8:** 1–10.
Holland, P.C. 1986. Temporal determinants of occasion setting in feature-positive discriminations. *Anim. Learn. Behav.* **14:** 111–120.
———. 1989a. Acquisition and transfer of conditional discrimination performance. *J. Exp. Psychol. Anim. Behav. Process.* **15:** 154–165.
———. 1989b. Feature extinction enhances transfer of occasion setting. *Anim. Learn. Behav.* **17:** 269–279.
———. 1991. Transfer of control in ambiguous discriminations. *J. Exp. Psychol. Anim. Behav. Process.* **17:** 231–248.
Holland, P.C., Hamlin, P.A., and Parsons, J.P. 1997. Temporal specificity in serial feature-positive discrimination learning. *J. Exp. Psychol. Anim. Behav. Process.* **23:** 95–109.
Kandel, E.R. and Schwartz, J.H. 1982. Molecular biology of learning: Modulation of transmitter release. *Science* **218:** 433–443.
Katsov, A. and Clandinin, T.R. 2006. Insect vision: Remembering the shape of things. *Curr. Biol.* **16:** R369–R371.
Kim, J.J. and Fanselow, M.S. 1992. Modality-specific retrograde-amnesia of fear. *Science* **256:** 675–677.
Lachnit, H. and Kimmel, H.D. 1991. Extending the Rescorla-Wagner theory to account for transswitching. *Biol. Psychol.* **32:** 193–200.
Law, E., Nuttley, W.M., and van der Kooy, D. 2004. Contextual taste cues modulate olfactory learning in *C. elegans* by an occasion-setting mechanism. *Curr. Biol.* **14:** 1303–1308.
Liu, L., Wolf, R., Ernst, R., and Heisenberg, M. 1999. Context generalization in *Drosophila* visual learning requires the mushroom bodies. *Nature* **400:** 753–756.
Liu, G., Seiler, H., Wen, A., Zars, T., Ito, K., Wolf, R., Heisenberg, M., and Liu, L. 2006. Distinct memory traces for two visual features in the *Drosophila* brain. *Nature* **439:** 551–556.
Matsumoto, Y. and Mizunami, M. 2004. Context-dependent olfactory learning in an insect. *Learn. Mem.* **11:** 288–293.
Miller, R.R. and Oberling, P. 1998. Analogies between occasion setting and Pavlovian conditioning. In *Occasion setting: Associative learning and cognition in animals* (eds. N.A. Schmajuk and P.C. Holland), pp. 3–35. American Psychological Association, Washington, D.C.
Murphy, G.G. and Glanzman, D.L. 1999. Cellular analog of differential classical conditioning in *Aplysia*: Disruption by the nmda receptor antagonist dl-2-amino-5-phosphonovalerate. *J. Neurosci.* **19:** 10595–10602.
Myers, C.E. and Gluck, M.A. 1994. Context, conditioning, and hippocampal rerepresentation in animal learning. *Behav. Neurosci.* **108:** 835–847.
Pearce, J.M. 1987. A model for stimulus generalization in Pavlovian conditioning. *Psychol. Rev.* **94:** 61–73.
———. 1994. Similarity and discrimination: A selective review and a connectionist model. *Psychol. Rev.* **101:** 587–607.
Pearce, J.M. and Bouton, M.E. 2001. Theories of associative learning in animals. *Annu. Rev. Psychol.* **52:** 111–139.
Rescorla, R.A., Grau, J.W., and Durlach, P.J. 1985. Analysis of the unique cue in configural discriminations. *J. Exp. Psychol. Anim. Behav. Process.* **11:** 356–366.
Rogers, R.F. and Matzel, L.D. 1996. Higher-order associative processing in *hermissenda* suggests multiple sites of neuronal modulation. *Learn. Mem.* **2:** 279–298.
Rogers, R.F., Schiller, K.M., and Matzel, L.D. 1996. Chemosensory-based contextual conditioning in *hermissenda crassicornis*. *Anim. Learn. Behav.* **24:** 28–37.
Schmajuk, N.A., Lamoureux, J.A., and Holland, P.C. 1998. Occasion setting: A neural network approach. *Psychol. Rev.* **105:** 3–32.
Schubert, M., Lachnit, H., Francucci, S., and Giurfa, M. 2002. Nonelemental visual learning in honeybees. *Anim. Behav.* **64:** 175–184.
Schulz, R.A., Chromey, C., Lu, M.F., Zhao, B., and Olson, E.N. 1996. Expression of the d-mef2 transcription in the *Drosophila* brain suggests a role in neuronal cell differentiation. *Oncogene* **12:** 1827–1831.
Sutton, R.S. and Barto, A.G. 1998. Reinforcement learning. MIT Press, Cambridge, MA.
Swartzentruber, D. 1991. Blocking between occasion setters and contextual stimuli. *J. Exp. Psychol. Anim. Behav. Process.* **17:** 163–173.
Sweeney, S.T., Broadie, K., Keane, J., Niemann, H., and O'Kane, C.J. 1995. Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron* **14:** 341–351.
Tang, S. and Guo, A. 2001. Choice behavior of *Drosophila* facing contradictory visual cues. *Science* **294:** 1543–1547.
Tang, S.M., Wolf, R., Xu, S.P., and Heisenberg, M. 2004. Visual pattern recognition in *Drosophila* is invariant for retinal position. *Science* **305:** 1020–1022.
Walters, E.T. and Byrne, J.H. 1983. Associative conditioning of single sensory neurons suggests a cellular mechanism for learning. *Science*

219: 405–408.
Wickens, D.D. 1987. The dual meanings of context: Implications for research, theory and applications. In: *Memory and learning: The Ebbinghaus centennial conference* (eds. D.S. Gorfein and R.R. Hoffman) pp. 135–152. Lawrence Erlbaum Associates, Inc., Hillsdale, NJ.
Wiener, J. 2000. Kontext-generalisierung in *Drosophila melanogaster*. Diploma thesis, Julius-Maximilians Universität, Würzburg, Germany.
Wilson, P.N. and Pearce, J.M. 1990. Selective transfer of responding in conditional discriminations. *Q. J. Exp. Psychol. B* **42:** 41–58.
Wolf, R. and Heisenberg, M. 1991. Basic organization of operant behavior as revealed in *Drosophila* flight orientation. *J. Comp. Physiol. [A]* **169:** 699–705.
———. 1997. Visual space from visual motion: Turn integration in tethered flying *Drosophila*. *Learn. Mem.* **4:** 318–327.
Wolf, R., Wittig, T., Liu, L., Wustmann, G., Eyding, D., and Heisenberg, M. 1998. *Drosophila* mushroom bodies are dispensable for visual, tactile and motor learning. *Learn. Mem.* **5:** 166–178.
Young, M.E., Johnson, J.L., and Wasserman, E.A. 2000. Serial causation: Occasion setting in a causal induction task. *Mem. Cognit.* **28:** 1213–1230.
Zars, T., Fischer, M., Schulz, R., and Heisenberg, M. 2000. Localization of a short-term memory in *Drosophila*. *Science* **288:** 672–675.

Received May 14, 2006; accepted in revised form July 13, 2006.

Research

Different parameters support generalization and discrimination learning in *Drosophila* at the flight simulator

Björn Brembs[1,3] and Natalie Hempel de Ibarra[2]

[1]*Institute of Biology and Neurobiology, Freie Universität Berlin, 14195 Berlin, Germany;* [2]*School of Life Sciences, University of Sussex, Falmer, Brighton, BN1 9QG, United Kingdom*

We have used a genetically tractable model system, the fruit fly *Drosophila melanogaster* to study the interdependence between sensory processing and associative processing on learning performance. We investigated the influence of variations in the physical and predictive properties of color stimuli in several different operant-conditioning procedures on the subsequent learning performance. These procedures included context and stimulus generalization as well as color, compound, and conditional discrimination (colors and patterns). A surprisingly complex dependence of the learning performance on the colors' physical and predictive properties emerged, which was clarified by taking into account the fly-subjective perception of the color stimuli. Based on estimates of the stimuli's color and brightness values, we propose that the different tasks are supported by different parameters of the color stimuli; generalization occurs only if the chromaticity is sufficiently similar, whereas discrimination learning relies on brightness differences.

All animals extract relevant cues from the continuum of the incoming sensory stream to learn about their environment and how to behave in it. But how are the salient, predictive cues extracted from this stream and what factors determine the composition of a memory template? Obviously, some things are learned faster and remembered better than others. The relative timing of stimuli is of course paramount (for discussion, see Brembs and Wiener 2006). Another factor could be the physical make-up of a predictive stimulus. For example, it is usually assumed that a conspicuous, localized stimulus will be easier to learn than a diffuse, extended background stimulus. But is this seemingly straightforward insight true for all sorts of memory templates? In this study, we use colors and patterns in the visual learning paradigms for *Drosophila melanogaster* at the flight simulator to approach this problem.

There is only limited evidence that *Drosophila* uses and learns color as visual cue (Quinn et al. 1974; Spatz et al. 1974; Menne and Spatz 1977; Bicker and Reichert 1978; Desalomon and Spatz 1983). On the other hand, colors have been used as stimuli in a number of studies involving visual-discrimination learning in the flight simulator (Wolf and Heisenberg 1997; Wolf et al. 1998; Brembs and Heisenberg 2001; Tang and Guo 2001), where visual patterns are presented on the inner wall of a vertical cylinder (arena) surrounding the tethered fly. The yaw torque signal generated by the fly can rotate the arena such that the animal can stabilize the panorama and choose flight direction with respect to the patterns. The coloration of the arena as pattern background can be changed by passing the light through appropriate filters before it reaches the arena (Fig. 1A). While there is a large body of work concerning the processing and learning of patterns in the arena (e.g., Wolf and Heisenberg 1991, 1995, 1997, 1998; Wolf et al. 1992, 1998; Dill et al. 1993, 1995; Dill and Heisenberg 1995; Heisenberg 1995; Guo et al. 1996; Guo and Götz 1997; Xia et al. 1997a,b, 1999; Gong et al. 1998; Liu et al. 1998, 1999; Wang et al. 1998, 2003; Ernst and Heisenberg 1999; Brembs and Heisenberg 2000, 2001; Heisenberg et al. 2001; Tang and Guo 2001; van Swinderen and Greenspan 2003; Greenspan and van Swinderen 2004; Tang et al. 2004; Guo and Guo 2005), very little is known about the processing of the colors. After the initial discovery that flies learn colors in the flight simulator (Wolf and Heisenberg 1997), Liu et al. (1999) used background coloration as a context cue during pattern-discrimination learning and found that context generalization depends critically on the spectra of the colors used. Specifically, if the spectra of the two background colors used as context did not overlap fully, flies did not generalize pattern memory between them, whereas colors with full spectral overlap supported context generalization.

Brembs and Heisenberg (2001) studied the effects of combining colors and patterns in compound stimuli that flies were able to learn. In a chance discovery, we now found a pair of color stimuli with very peculiar effects ("Rosco" blue and green; Fig. 1B). When these colors were presented as background together with black patterns, such a compound of cues was not learned by the flies (Fig. 1C,D). Usually, with two cues as predictive stimuli, such situations can be solved very well by the flies (Brembs and Heisenberg 2001). It is important to emphasize that the patterns alone are sufficient predictors, so the flies could disregard the colors and still be able to solve the task. Even more curiously, if after compound training the pattern memory was tested without the "Rosco" colors, it appeared as if it had only been suppressed by the presence of the colors (Fig. 1E). Interestingly, the spectra of these colors overlap only partially, whereas those used previously did either overlap fully or did not overlap at all. This provided us with an excellent opportunity to systematically characterize the relationship between the physical properties of the colors and the associative processes underlying color learning. Inspired by the conclusions from our companion paper, we decided to study the colors in two different generalization tasks and in three discrimination tasks (Fig. 1F) by setting them up as context, conditioned stimuli (CS), and as occasion setters (OS) (Brembs and Wiener 2006).

[3]Corresponding author.
E-mail bjoern@brembs.net; fax 49-308-385-5455.
Article is online at http://www.learnmem.org/cgi/doi/10.1101/lm.319406.

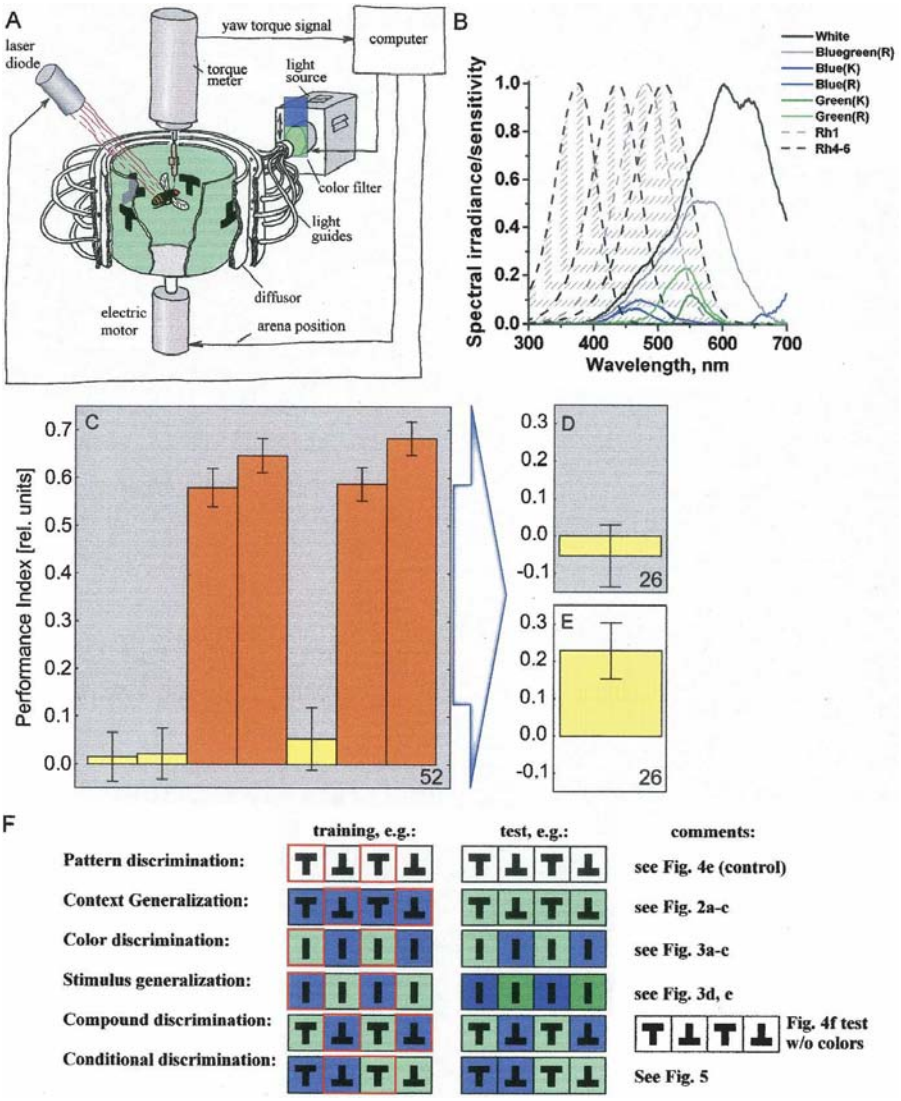


Figure 1. Flight simulator set-up and experimental schematics. (*A*) The fly is flying stationarily in a cylindrical arena homogeneously illuminated from behind. The fly's tendency to perform left or right turns (yaw torque) is measured continuously and fed into the computer. The computer controls arena position, IR-laser (heat) activation and color of illumination according to the conditioning rules. (*B*) Spectral irradiance of the arena illumination using different color filters. Note the lack of overlap for the Kodak filters [Blue(K), Green(K)], the partial overlap for the Rosco filters [Blue(R), Green(R)] and the full overlap for either of the Kodak filters with the (Schott) BG18-like Rosco #5433 filter [Bluegreen (R)], respectively. Spectral sensitivity of rhodopsins is shown for the receptors R1 (Rh1), R7 (Rh5), and R8 (Rh4, Rh6), which are predominantly excited by the used illuminations. (*C*) Course of experiment. Bars show performance indices (PI) of successive 2-min intervals of pretest (yellow bars; PI_1, PI_2) and training (orange bars; PI_3, PI_4, PI_6, PI_7). Animals are trained to learn a pattern/color compound. The animals are then divided in two groups for testing. (*D*,*E*) PI of the first 2-min. test period after the last training (PI_8). (*D*) Compound test after compound training. (*E*) Testing for pattern memory after compound training. (*F*) Experimental schematics. Patterns and colors depict the wall of the cylinder surrounding the fly. Colored boxes indicate the four 90° quadrants. All of the three filter pairs were used, but only blue and green are depicted here as examples. Red squares in the example color/pattern schematics depict heated quadrants. Note that even though adjacent quadrants may be drawn in different colors here, the illumination of the entire arena is always changed. Although the original pattern discrimination learning experiment is performed without any color filters, pattern learning still takes place if the spectrum of the lamp is restricted by color filters. A test for context generalization takes place when the color of the illumination is changed between training and test. Flies can be trained to discriminate colors instead of patterns by changing the illumination whenever the fly changes flight direction from one quadrant to the next. Stimulus generalization is tested by training the flies in the color-learning paradigm with one pair of filters and testing them with a second pair of filters. Combining colors and patterns to a compound discrimination paradigm enables the researcher to test for the effects of colors on pattern learning (see *C* and *D*). Finally, the conditional discrimination paradigm tests the ability of the colors to convey information about the pattern/heat contingency. See Materials and Methods for a detailed description.

Results

It has been established previously that flies that have been trained to discriminate between two visual patterns with background illumination in one color and tested for context generalization by presenting the same patterns in a different color background do not show the conditioned pattern discrimination when the spectrum of the test color does not overlap with that of the training color (Fig. 2A; from Liu et al. 1999). However, if the spectrum of one of the colors is fully contained within the spectrum of the second (i.e., they exhibit full overlap of their spectra), the pattern memory is generalized across color contexts and in both directions of the reciprocal arrangements (Fig. 2C; Liu et al. 1999). Interestingly, when the flies are trained in one color and tested in another, the spectrum of which partially overlaps with that of the training color, flies again do not show the conditioned discrimination (Fig. 2B). Thus, colors with nonoverlapping or only partially overlapping spectra do not support context generalization. This effect cannot be attributed to the colors themselves, as patterns are used by the flies for the conditioned avoidance when the colors are kept constant throughout the experiment (Fig. 2D). Importantly, these experiments show that the flies distinguish the two colors with partially overlapping spectra; otherwise the flies would show the conditioned pattern preference in the new color. Apparently, lack of discrimination cannot be the explanation for the failure to learn the pattern/color compound cue (Fig. 1D).

However, to get an idea of the degree to which the colors in the three color pairs differ, we compared how flies discriminate color pairs with no, partial, or complete spectral overlap when they are set up as operant CSs (see Fig. 1C). While colors with full or no overlap in their spectra can be discriminated very well (Fig. 3A,C), flies do not show conditioned discrimination after training with colors that show only partial overlap in their spectra (Fig. 3B). From these results alone, one usually would conclude that flies cannot discriminate colors with partial overlap of their spectra. But the context generalization experiments suggest the opposite. One hypothesis explaining these contradictory results obtained with the partially overlapping colors may be that colors with partially overlapping spectra can be distinguished by the flies but not sufficiently as to support discrimination learning. To test this hypothesis, we

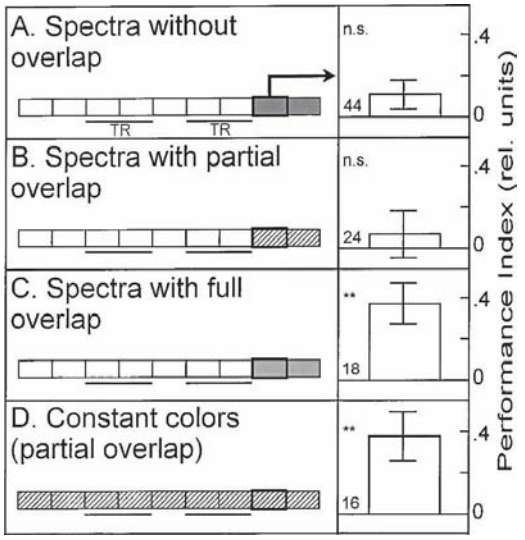


Figure 2. Context generalization. (*A,B,C*) Training of patterns in one color, test in the second. Change in hatching or shading of the boxes denotes change in background illumination. (*A*) Context generalization between colors with nonoverlapping spectra. Arena coloration changes after period 7. Dark-gray boxes denote the change between colors with nonoverlapping spectra. No significant learning score was obtained. Data from Liu et al. (1999). (*B*) Context generalization between colors with partially overlapping spectra. Hatched boxes denote the periods in which the colors were changed. No significant learning score was obtained. (*C*) Context generalization between two colors with fully overlapping spectra. Light-gray boxes depict the periods of changed arena coloration. Only the color pair with full spectral overlap supports context generalization. (*D*) Pattern learning is unaffected if the background coloration is kept constant. Hatched boxes depict constant illumination with one of the two colors (the pair with partial spectral overlap) throughout the experiment (no color change). White, hatched, or gray boxes denote 2-min experimental periods. White boxes denote periods in one color; hatched or gray boxes indicate a change of color from one to the other of the pair. (Dark gray) No spectral overlap; (hatched) partial spectral overlap; (light gray) full spectral overlap. The performance indices of the highlighted test periods (bold) are displayed in the bar graphs on the *right*. $^{**}P < 0.01$; (n.s.) not significant. Numbers next to bar graphs indicate number of animals. Lines *under* experimental periods (indicated by "TR") denote training periods. Performance index: $PI = (t_a\text{-}t_b)/(t_a+t_b)$.

asked whether flies can generalize a conditioned discrimination between partially overlapping colors to the pair with nonoverlapping spectra and vice-versa (Fig. 3; see Fig. 1). The prediction was that if flies only distinguish the partially overlapping colors, but do not learn them, we should not find any generalization. If, on the other hand, the flies can both distinguish and learn the partially overlapping colors, we may find generalization from the partially overlapping colors to the nonoverlapping colors. Indeed, we found stimulus generalization, but only in one direction; when colors with no overlap are trained (i.e., to discriminate between Kodak green and blue) and then the flies are tested with the partially overlapping color pair (i.e., whether they discriminate Rosco green and blue), no significant performance index is obtained (Fig. 3D). However, if the inverse situation is invoked, the flies trained to distinguish partially overlapping colors show a generalized conditioned discrimination. The flies preferred the unpunished color of the nonoverlapping color pair during the test phase (i.e., Kodak blue if Rosco green was punished and vice versa; Fig. 3E). In conclusion, the flies discriminate partially overlapping colors and generalize their conditioned color preference to the nonoverlapping colors. However, retrieval of the conditioned preference is not directly guided by the perceptual difference between the partially overlapping colors. Following the same line of argument, we can conclude that flies acquired a conditioned color preference even during color and pattern-color compound discrimination training with partially overlapping colors, but failed to retrieve this preference with these colors.

Combining operant pattern and color-discrimination learning to compound-discrimination learning (see also Fig. 1C,D,E), we studied the interaction of the two stimuli (Fig. 4). The results mimic those obtained in the color-discrimination experiments, i.e., colors with full or no overlap in their spectra support compound discrimination (Fig. 4A,C), whereas flies do not show conditioned discrimination after compound training in which the patterns were presented together with background colors that show partial overlap in their spectra (Fig. 4B). It needs to be pointed out that training the flies with the patterns alone, i.e., on a background illuminated by white light without color filters, is sufficient to enable the flies to choose the right flight direction (Fig. 4E). In other words, the successive presence of the colors with partially overlapping spectra disrupts the pattern discrimination normally taking place. Importantly, it is not the spectral restriction per se that disrupts pattern discrimination, as pattern-discrimination learning is evident if the background is colored in one of the two overlapping colors, but kept constant (Figs. 2D, 4D). An important control procedure is to remove the overlapping color filters after compound training, presenting the patterns in white light. Flies expressed a significant pattern prefer-

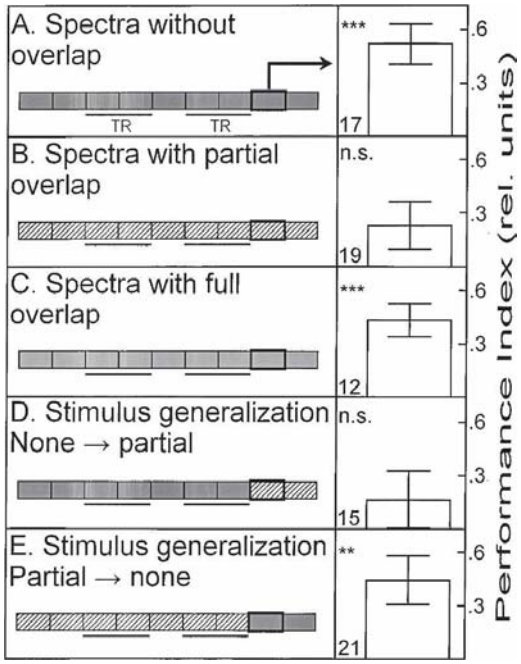


Figure 3. Color discrimination learning and stimulus generalization across different colors as CSs. (*A,B,C*) Color discrimination learning with the three color pairs. Animals were trained to avoid flight directions associated with one of the two arena colorations. (*D,E*) Reciprocal stimulus generalization between the color pair with partial and the one with no spectral overlap. Training one color pair and testing the other only yields a significant learning score in one direction: If the colors with partial overlap are trained, the animals show conditioned color preference only with the nonoverlapping color pair. Hatched or gray boxes denote 2-min experimental periods. (Dark gray) No spectral overlap; (hatched) partial spectral overlap; (light gray) full spectral overlap. The performance indices of the highlighted test periods (bold) are displayed in the bar graphs on the *right*. $^{***}P < 0.001$; $^{**}P < 0.01$; (n.s.) not significant. Numbers *next* to bar graphs indicate number of animals. Lines *under* experimental periods (indicated by "TR") denote training periods. Performance index: $PI = (t_a\text{-}t_b)/(t_a+t_b)$.

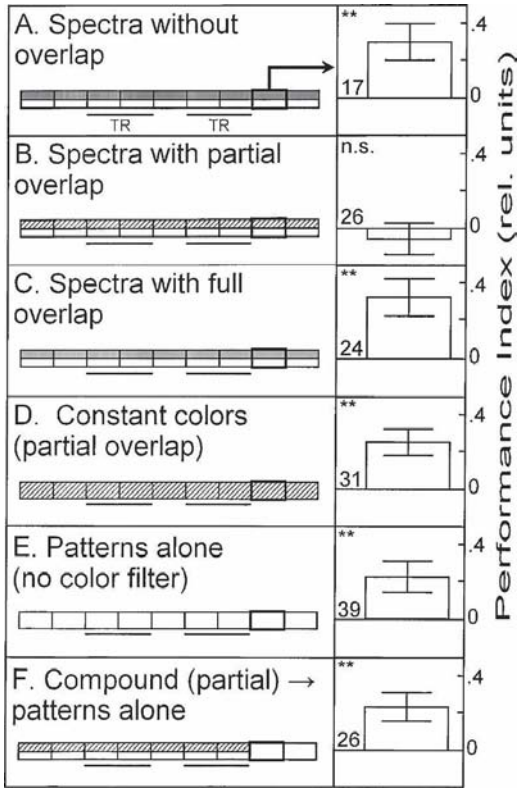


Figure 4. Color and pattern compound discrimination learning. (*A,B,C*) Pattern/color compound discrimination learning with the three color pairs. (*B*) Same data as Figure 1D. (*D*) Pattern discrimination learning in constant colors (partial overlap; identical experiment as in Fig. 2D). (*E*) Pattern discrimination learning without colored background (no color filters). (*F*) Training in pattern/color compound (partial overlap as in *C*) and final test with the color filter removed (training as in Fig. 1C; same data as Fig. 1E). Boxes with hatched upper and white lower half denote periods with color filters (partial overlap) and patterns; entirely white boxes denote periods without arena coloration and only patterns. Patterns were upright and inverted T's in all cases. Boxes denote 2-min experimental periods. Dark gray denotes colors without overlap, hatching denotes colors with partial overlap, and light gray denotes colors with full spectral overlap. Boxes with a white lower half denote compound presentation of colors and patterns. Entirely white boxes denote periods where no color filters were present (patterns only). The performance indices of the highlighted test periods (bold) are displayed in the bar graphs on the *right*. $^{**}P < 0.01$; (n.s.) not significant. Numbers *next* to bar graphs indicate number of animals. Lines *under* experimental periods (indicated by "TR") denote training periods. Performance index: PI = $(t_a\text{-}t_b)/(t_a+t_b)$.

ence during this test, revealing the dominant effect of the partially overlapping colors in the retrieval of conditioned pattern preferences (Fig. 4F).

Finally, to provide further evidence that our results are task (discrimination vs. generalization) and not paradigm specific, we tested the latest and most complex paradigm at the flight simulator, conditional discrimination, a form of occasion setting (see Fig. 1). In this paradigm, the colors serve as a higher-order predictor, indicating the nature of the pattern/heat contingency (Brembs and Wiener 2006). Specifically, background coloration of one color indicates that the upright T is being punished and the other color indicates that the inverted T is being punished. Again paralleling our previous results, both colors with completely overlapping spectra and colors with nonoverlapping spectra support conditional discrimination (Fig. 5A,C), whereas colors with partially overlapping spectra do not (Fig. 5B).

Puzzled by this unexpected complexity in our results, we decided to characterize the colors from the fly's perceptual point of view (Table 1). The receptors R1–R6 mediate achromatic coding of visual information, whereas R7 and R8 encode chromaticity. The non- and fully overlapping colors showed a large difference in quantum catches for the R1–R6 receptors, thus being clearly different in brightness for the flies, whereas the partially overlapping colors were not (Table 1).

Estimation of chromaticity is more difficult. It is generally agreed that R7 and R8 receptors feed into color-coding mechanisms; however, the exact contribution of the two subtypes of R7 and R8 receptors belonging to two ommatidial types is still unknown. Experiments by Troje (1993) and Fukushi (1985, 1989) with *Lucilia* indicate that both subtypes may be involved. Since in our experiments the UV range was not used, the R8 receptors were the most strongly excited ones (Fig. 1). We calculated chromaticities using the input of either all four receptor types (Table 1, R7–R8) or alternatively discarding any signal of the very weakly excited R7 receptor with Rh3 opsin (Table 1, Rh4–Rh6). Also, we looked at the predictions of a hypothesized ommatidial opponency mechanism for each of the R7/R8 combinations following an assumption of the processing model by Troje (1993). Predictions arising from these calculations are not uniform (Table 1). The best correlation to the behavioral results is achieved by calculating color differences from the three strongly excited receptors (Rh4–Rh6) and the ommatidia carrying the Rh4/Rh6 combination. Smaller distances are predicted and calculated for spectra of similar shape that were generalized by the flies, such as the Blue Rosco and Blue Kodak, Green Rosco and Green Kodak, or the other fully overlapping spectra. Larger distances are calculated for spectra of dissimilar shape, e.g., non- and partially overlapping colors, which were not generalized by the flies.

Classifying our stimuli according to the two perceptual qualities, we establish two subjective axes (Fig. 6); color pairs line up on a brightness difference gradient, in which the partially overlapping colors differ the least in brightness, the nonoverlap-

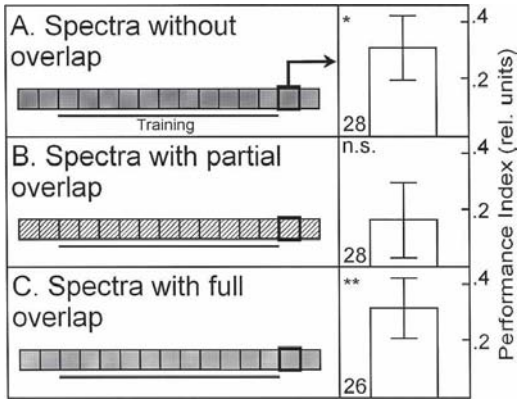


Figure 5. Conditional discrimination with the three different color pairs. (*A*) Conditional discrimination experiment where the spectra of the two colors indicating the nature of the pattern/heat contingency did not overlap (dark-gray boxes). (*B*) Conditional discrimination using colors with partially overlapping spectra (hatched boxes). (*C*) Conditional discrimination experiment in which the spectra of the two colors overlapped fully (light-gray boxes). Hatched or gray boxes denote 1-min experimental periods. (Dark gray) No spectral overlap; (hatched) partial spectral overlap; (light gray) full spectral overlap. The performance indices of the highlighted test periods (bold) are displayed in the bar graphs on the *right*. $^{**}P < 0.01$; $^{*}P < 0.05$; (n.s.) not significant. Numbers next to bar graphs indicate number of animals. Lines *under* experimental periods (indicated by "Training") denote training periods. Performance index: PI = $(t_a\text{-}t_b)/(t_a+t_b)$.

Table 1. Differences in the fly-subjective brightness and chromaticity of the background colors

	Difference in chromaticity (RNL units)						in brightness (Log units)	
	R7–R8	Rh3/5		Rh4–Rh6	Rh4/6		R1–R6	
Nonoverlapping spectra								
1. Blue (Kodak), Green (Kodak)	26.0	17.1	Large	19.4	15.5	Large	1.52	Large
Partially overlapping spectra								
2. Blue (Rosco), Green (Rosco)	15.5	3.42	Medium/ Small	15.4	14.2	Large	0.07	Small
Fully overlapping spectra								
3. Blue (Kodak), Bluegreen (Rosco)	13.1	1.4	Medium/ Small	12.0	11.1	Medium	1.77	Large
4. Green (Kodak), Bluegreen (Rosco)	19.7	18.5	Large	11.0	4.4	Medium/ Small	3.29	Large
Permutations (full overlap)								
5. Blue (Rosco), Blue (Kodak)	7.2	3.3	Small	5.9	5.3	Small	0.65	Medium
6. Green (Rosco), Green (Kodak)	17.8	16.9	Large	11.2	4.1	Medium/ Small	2.08	Large

Animals were trained to discriminate and generalize between colors presented alone or as background for black T-shaped patterns. Colors had either nonoverlapping (1), partially overlapping (2), or completely overlapping spectra (3/4). Brightness differences were estimated through the receptor signals of R1–R6 receptors. All colors presented in sequence differed strongly in brightness, except those with the partially overlapping spectra (2). Chromaticity was computed for different input of R7 and R8 receptor signals. The second column shows values characterizing the color differences using input from all four spectral R7 and R8 receptor types. Note the larger the value, the better colors would be distinguished by the flies. The third and fifth columns (Rh3/Rh5 and Rh4/Rh6) refer to hypothetic single-opponent mechanisms based on the input from the R7/R8 pair in two ommatidial subtypes. The fifth column (Rh4–Rh6) shows the results of calculating the chromatic input from the three predominantly excited receptor types. The behavioral data are closest to the color differences as predicted from the opponency signal of the central photoreceptors of the Rh4/Rh6 ommatidial type and coherently from the Rh4–Rh6 input being dominated by the signals from Rh4/Rh6 ommatidia. For instance, flies generalized between spectra of similar shape (3–6) and did not generalize between spectra with dissimilar shape (1–2), and also have larger chromatic differences than spectral pairs in 3/4 and 5/6.

ping pair differs more, and the fully overlapping pairs differ most in brightness (Fig. 6A). Along the chromaticity axis, the pairs line up with the fully overlapping color pairs showing the smallest chromaticity difference, the partially overlapping pair showing clearly more difference, and the nonoverlapping pair having the largest chromaticity difference (Fig. 6B).

Discussion

In this study, we characterized the functional relationship between the physical properties of three sets of color stimuli and the associative processes underlying color learning in two generalization and three discrimination learning tasks. We found that the color pair with partially overlapping spectra had a number of surprising properties. These colors do not prevent the acquisition of pattern memory, but rather the retrieval of it. Moreover, the partially overlapping colors can be distinguished and learned, but the learned preference cannot be retrieved with these colors present. Judging from all three color pairs' spectra alone, one would classify the nonoverlapping one as most different, the fully overlapping colors as most similar, and the partially overlapping colors somewhere in-between. One would expect to find a fairly simple system, where generalization and discrimination are steady functions of similarity with inverted signs. Instead, we found a complex set of results that were highly dependent on the spectral properties of the colors used, but where the physical properties alone could not explain all of the variability.

The generalization experiments are in line with the simple expectations; only the color pairs classified as most similar (the ones with full spectral overlap) support the generalization of pattern memory across two contexts characterized by these colors (Fig. 2C). Context generalization was not detected if the background colors were characterized by partially overlapping spectra, indicating that these colors can be distinguished (Fig. 2B). Moreover, color memory acquired during training with these partially overlapping colors alone can generalize to colors without spectral overlap (Fig. 3E), the spectra of which are fully contained within the spectra of the partially overlapping colors. However, the simple predictions are not met in the discrimination experiments. The same colors (with partial overlap), although being distinguishable, do not support conditioned dis-

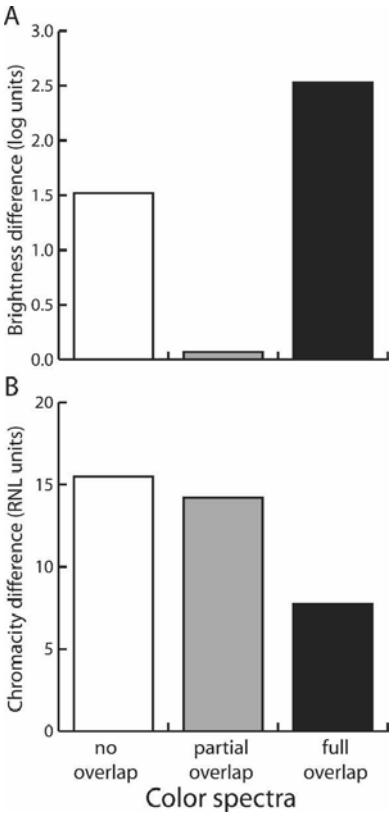


Figure 6. Subjective brightness and chromaticity differences between the three different color pairs. (*A*) Subjective brightness estimates reveal almost no difference between the two partially overlapping colors, while the other two pairs differ considerably. (*B*) Subjective chromaticity estimates reveal similar chromaticity differences between the two narrowband pairs (no overlap and partial overlap), whereas the chromaticity of the fully overlapping color pairs is more similar. (White bars) Color pair with nonoverlapping spectra; (gray bars) color pair with partially overlapping spectra; (black bars) color pair with fully overlapping spectra.

crimination (Figs. 3B, 5B) and even prevent the retrieval of pattern memory when they are combined with the patterns during compound conditioning (Fig. 4B,F). In contrast, both the most similar colors (with full overlap) and the most different colors (without overlap) support all of our discrimination learning tasks.

This demonstrates the interaction between sensory processing (distinguishing between the colors) and associative processing (forming a memory template); in the context generalization experiment, the partially overlapping colors are incorporated into the memory template and prevent generalization (much like the nonoverlapping colors), but in the discrimination tasks (color and compound discrimination learning, conditional discrimination) they are not sufficiently incorporated to support retrieval of the memory. Yet, colors that are not incorporated into the memory template in a context generalization experiment (i.e., the colors with fully overlapping spectra) support discrimination learning just fine.

Hoping that the key to understanding such complicated results may lie in the subjective perceptual quality of color stimuli, we computed the flies' perception of the colors (Fig. 6). The percept of a color is influenced by the physical function of light intensity and wavelength distribution. These basic properties can be encoded as brightness and color cues, which are commonly processed in parallel neural systems and mediate different perceptual functions (Livingstone and Hubel 1988; Gegenfurtner and Kiper 2003; Osorio and Vorobyev 2005). Thus, colors carry both chromaticity and brightness cues that may result in a unique percept, but can also mediate different parts of the behavioral output (Lehrer 1987; Hempel de Ibarra et al. 2002; Kelber et al. 2003; Kelber 2005). From such computations (Fig. 6), we derive the most parsimonious hypothesis that *Drosophila* extracts different spectral parameters from the color stimuli to solve the generalization and the discrimination tasks, respectively. For generalization across two colors, they must be sufficiently similar in their chromaticity. In the fully overlapping color pairs, which are the most similar in terms of chromaticity, generalization occurs despite even relatively large brightness differences (Fig. 2C). Larger differences in chromaticity are sufficient to prevent generalization, despite only small brightness differences and some spectral overlap (Fig. 2B). So far, these results conform to the simple "similarity" expectation. For conditioned discrimination of two colors (Figs. 3–5), neither the spectral overlap, nor the chromaticity difference, nor the strength of background brightness with a stronger or weaker contrast to the black pattern can account for our complex set of results. Instead, it appears that there has to be a large brightness difference for two colors to support any of our kinds of discrimination learning. Colors with only partial spectral overlap, large color differences, but only small brightness differences can be distinguished by the flies (Figs. 2B, 3E), but spoil the test (Figs. 3B, 5B) and prevent the retrieval of pattern memory (Fig. 4B,F). Apparently, similarity in brightness is sufficient to prevent the three kinds of discrimination learning used in this study, even if other parameters differ widely. Interestingly, the chromaticity difference between the two partially overlapping colors can be learned, but it can only be retrieved with colors that differ also in brightness (Fig. 3E). Thus, the failure in discrimination learning of partially overlapping colors is attributable to a failure in retrieval, rather than acquisition.

These results are intriguing with respect to the results described in our companion paper (Brembs and Wiener 2006). Their data indicate that the predictive relationship to the reinforcer is the decisive factor of whether a context is incorporated into the memory template (context dependence or discrimination) or not (context independence or generalization). Our data suggest that extraction of different parameters of the background stimulus (context) underlies generalization and discrimination, respectively. One might speculate also that different neural substrates support generalization and discrimination, respectively, and that it is the combination of physical and predictive properties of the "context" that determines whether or not any given memory will be context dependent or independent. In this view, it makes little sense to study context dependence (discrimination) or independence (generalization) without determining the source of the phenomenon in terms of the physical (this study) and the predictive (Brembs and Wiener 2006) properties of the stimuli in question.

Materials and Methods

Flies

Flies are kept on standard cornmeal/molasses medium (Guo et al. 1996) at 25°C and 60% humidity with a 14-h light/10-h dark regime. Females aged 24–48 h are briefly immobilized by cold anesthesia and glued (Loctite UV glass glue) with head and thorax to a triangle-shaped copper hook (diameter 0.05 mm) the day before the experiment. The animals are then kept individually overnight in small moist chambers containing a few grains of sucrose.

Spectral stimuli

Three pairs of color filters were used (see Fig. 1B). (1) Filters with nonoverlapping spectra—broad-band blue (No. 47) and broad-band green (No. 99) Kodak Wratten gelatin filter. (2) Filters with partially overlapping spectra—Rosco "just blue" (No. 079) and Rosco "dark green" (No. 124). (3) Filters with fully overlapping spectra—"Daylight" blue-green (Rosco "surfblue" No. 5433) with either the Kodak green or the Kodak blue filter. The transmission spectrum of the Rosco blue-green filter used in this study is equivalent to that of the BG18 filter (Schott, Mainz) used by Liu et al. (1999) (data not shown). Light spectra were measured inside the arena using a calibrated photospectrometer (SD 2000, Ocean Optics). To calculate photoreceptor excitations as integral of the spectrum of a white light source filtered through different color filters and spectral receptor sensitivities (Wyszecki and Stiles 1982), we used template-absorbance spectra (Stavenga et al. 1993) of *Drosophila* rhodopsins for its known sensitivity peaks (λmax of 480, 347, 375, 436, and 508 nm, Rh1 and Rh3–Rh6, respectively) (Feiler et al. 1988, 1992; Salcedo et al. 2003). Chromatic differences of the color stimuli were determined using the Receptor Noise Limited model of color vision (Vorobyev and Osorio 1998; Hempel de Ibarra et al. 2000), which has been successfully applied to tri- or tetrachromatic visual systems of human, birds, and bees, respectively (Vorobyev and Osorio 1998; Hempel de Ibarra et al. 2000). The input to color-opponent mechanisms was assumed to be either through all four R7 and R8 receptor cells or separately through each of the two R7/R8 units belonging to different ommatidia (Rh3/Rh5, Rh4/Rh6) (Chou et al. 1999). The UV range was excluded in our light stimuli, thus Rh3 was hardly excited at all, which allows calculation of a trichromatic input from Rh4–Rh6. Since the presented color pairs were well above their discrimination thresholds, a common value was used as noise estimate in R7/R8 receptor cells (Weber fraction of 0.1) (c.f. Vorobyev et al. 1998, 2001). We assumed its independence from the spectral channel and included a differential input of receptors based on a distribution ratio of 1:2.4 of the different ommatidia for the two ommatidial types Rh3/Rh5 and Rh4/Rh6 (Stark and Thomas 2004). Fly-subjective brightness was estimated through the quantum catch of the R1–R6 (Rh1) receptors (Heisenberg and Buchner 1977; Hardie 1986; Anderson and Laughlin 2000). The perceptual differences of the color stimuli are listed in Table 1.

Apparatus

The *Drosophila* flight simulator is a computer-controlled feedback system; the fly uses its yaw torque to control the rotations of a

panorama surrounding it. The core device is the torque meter (Götz 1964; Heisenberg and Wolf 1984), which measures a fly's angular momentum around its vertical body axis. The fly, glued to the hook, is attached to the torque meter via a clamp to accomplish stationary flight in the center of a cylindrical panorama (arena; diameter 58 mm) homogeneously illuminated from behind (Fig. 1A). The light source is a 100W, 12V tungsten-iodine bulb. For background coloration of the arena, the light is passed through one of the different filters described above. Filters can be exchanged by a fast solenoid within 0.1 sec.

A computer-controlled electric motor rotates the arena such that its angular velocity is proportional to, but directed against the fly's yaw torque (coupling factor $K = -11°/sec \cdot 10^{-10}$ Nm). This enables the fly to stabilize the panorama and to control its angular orientation. This virtual "flight direction" (i.e., arena position) is recorded continuously via a circular potentiometer (Novotechnik, A4102a306). An analog to digital converter card (PCL812; Advantech Co.) feeds arena position and yaw torque into a computer that stores the traces (sampling frequency 20 Hz) for later analysis.

Punishment is achieved by applying heat from an adjustable infrared laser (825 nm, 150 mW), directed from behind and above onto the fly's head and thorax. The laser beam is pulsed (~200 msec pulse width at ~4 Hz) and its intensity reduced to assure the survival of the fly.

General experimental design

Each fly is used only once. The time course of the experiment is divided into consecutive periods of either 1- or 2-min duration. Depending on whether heat is applied during such a period, it is termed a training period (heat on) or a test period (heat off). The treatment of the flies during these periods determines the type of experiment, as described below.

Discrimination learning—patterns

For patterns as CS (Wolf and Heisenberg 1991), four black, T-shaped patterns of alternating orientation (i.e., two upright and two inverted) are evenly spaced on the arena wall (pattern width $\psi = 40°$, height $\theta = 40°$, width of bars = 14°, as seen from the position of the fly). A computer program divides the 360° of the arena into four virtual 90° quadrants, the centers of which are denoted by the patterns. During training periods, heat punishment is made contiguous with the appearance of one of the pattern orientations in the frontal visual field. Reinforcement of each pattern is always equalized within groups. During test periods, the heat is permanently switched off (see Fig. 1C; pattern learning).

Discrimination learning—colors

For colors as CS (Wolf and Heisenberg 1997) the centers of the four virtual quadrants are denoted by four identical vertical stripes (width $\psi = 14°$, height $\theta = 40°$). The color of the illumination of the whole arena is changed whenever one of the virtual quadrant borders passes a point in front of the fly. During training periods, heat punishment is made contiguous with one of the colors. Reinforcement of each color is always equalized within groups. During test periods, the heat is permanently switched off (see Fig. 1C; color learning).

Discrimination learning—color/pattern compound

If a compound of patterns on a colored background is used as visual cue, the four T-shaped patterns are used and the color is changed as described (Brembs and Heisenberg 2001). During training periods, heat punishment is made contiguous with both the appearance of one of the pattern orientations in the frontal visual field and with the concomitant change in arena illumination. Reinforcement of each pattern/color is always equalized within groups. During test periods, the heat is permanently switched off (see Fig. 1C; compound discrimination).

Discrimination learning—conditional discrimination (occasion setting)

In this paradigm, arena coloration is used to indicate the nature of the pattern-heat contingency. For instance, flying toward the upright T is punished under green illumination and the inverted T is unpunished, but then blue illumination indicates the reverse pattern-heat contingency. In this experiment, neither of the stimuli alone can unambiguously predict reinforcement. Only the combination of the stimuli is predictive of the heat. In this paradigm, the flies control both colors and patterns operantly. The 360° of the arena are still divided into four virtual 90° quadrants as before. The center of each quadrant is also still denoted by the patterns (alternating upright and inverted Ts). The difference consists of the arrangement of color and heat with the quadrants. While heat was associated with two opposite quadrants (e.g., the ones with the upright T in the center) before, heat is now associated with adjacent quadrants (i.e., one with an upright and one with an inverted T). Thus, instead of being switched on or off at each of the four quadrant borders, the heat is now switched on or off at only two opposite borders. The color of the arena illumination is changed at the remaining two opposite quadrant borders, where the heat is not switched on or off. Thus, heat is applied in two quadrants, which include an upright and an inverted T as well as the quadrant border where the background coloration is changed. Conversely, arena coloration is changed exactly between the two punished patterns and between the two unpunished patterns. In such a way, heat is applied when the flies fly toward, say, a green upright T and a blue inverted T and switch the heat off by flying into one of the other two quadrants with a green inverted T and a blue upright T. One arrangement of quadrants may thus look as follows: The first quadrant features the upright T and whenever the fly enters this quadrant, the whole arena turns to blue illumination. The second quadrant features the inverted T and the arena illumination remains blue. If the fly enters the third quadrant with the upright T, the whole arena turns to green. In the fourth quadrant, the inverted T is in the center, but the arena illumination stays green. The heat regime is such that neither pattern nor color alone could predict punishment. For example, heat is switched on whenever the fly enters quadrants 2 or 3, but no heat is presented when entering quadrants 1 or 4. This heat regime is used for half of the animals, whereas the other half of the animals is not punished in quadrants 2 and 3, but quadrants 1 and 4 are punished (see Fig. 1C; conditional discrimination).

The training phase lasts 11 min and is divided into 1-min periods. After each period, the arena is set to a random position to minimize conditioning to spurious spatial cues. The spatial arrangement of patterns and colors was randomized across periods (i.e., if the patterns in quadrants 1 and 2 were "blue" and the patterns in quadrants 3 and 4 "green" in one period, this association was reversed in a random selection of other periods). This randomization minimized the spatial contingency and emphasized the logical contingency between patterns, heat, and colors. After 11 min of training, the animals are tested for 1 min for their quadrant preference with the heat permanently switched off.

Context generalization

Pattern discrimination training is conducted as described above, albeit with one of the color filters providing constantly colored background illumination of the entire arena. Following the original context generalization experiment by Liu et al. (1999), only one color change takes place after seven 2-min periods (2 × test, 2 × training, test, 2 × training), introducing a novel background color to the 2-min test period after the last training. For each color pair, the order of the training-test change in color is balanced across animals. A successful context generalization experiment is characterized by a positive learning score, which indicates that the pattern memory was generalized across the different color contexts. Such a successful experiment also shows that the pattern can be processed independently from the color, and the two stimuli are not perceived as a compound (Brembs and Heisenberg 2001). Context generalization is different from

context conditioning, where the animals learn to respond to a context. In this study, we never performed context conditioning, but only tested for the ability of a context change to disrupt the transfer of operant pattern memory between contexts. Successful context generalization is characterized by a continued conditioned pattern preference despite the context change (see Fig. 1C; context generalization).

Stimulus generalization

Color-discrimination training is conducted as described above. At the same point in the experiment as in context generalization, the color filters are exchanged to a different pair of filters. Then, color preference is tested with the heat permanently switched off, testing for color-discrimination learning during the 2-min test period after the last training (see Fig. 1C; stimulus generalization).

Data evaluation and statistics

The color and/or pattern preference of individual flies is calculated as the performance index: PI = $(t_a-t_b)/(t_a+t_b)$. During training periods, t_b indicates the time the fly is exposed to the heat and t_a the time without heat. During test periods, t_a and t_b refer to the times when the fly chose the formerly (or subsequently) unpunished or punished situation, respectively. Thus, a PI of 1 means the fly spent the entire period in the quadrants not associated with heat, whereas a PI of -1 indicates that the fly spent the entire period in the quadrants associated with heat. Accordingly, a PI of 0 indicates that the fly distributed the time evenly between heated and nonheated quadrants. PI's from test periods are called "test PIs" or "learning scores." Learning scores were tested for significance using a *t*-test for single means against zero, following Liu et al. (1999).

Acknowledgments

This work was supported by the DFG (BR 1893/3-2).

References

Anderson, J.C. and Laughlin, S.B. 2000. Photoreceptor performance and the co-ordination of achromatic and chromatic inputs in the fly visual system. *Vision Res.* **40:** 13–31.

Bicker, G. and Reichert, H. 1978. Visual learning in a photoreceptor degeneration mutant of *Drosophila melanogaster*. *J. Comp. Physiol.* **127:** 29–38.

Brembs, B. and Heisenberg, M. 2000. The operant and the classical in conditioned orientation in *Drosophila melanogaster* at the flight simulator. *Learn. Mem.* **7:** 104–115.

———. 2001. Conditioning with compound stimuli in *Drosophila melanogaster* in the flight simulator. *J. Exp. Biol.* **204:** 2849–2859.

Brembs, B. and Wiener, J. 2006. Context generalization and occasion setting: Mushroom bodies stabilize visual memory in *Drosophila*. *Learn. Mem.* (this issue).

Chou, W.H., Huber, A., Bentrop, J., Schulz, S., Schwab, K., Chadwell, L.V., Paulsen, R., and Britt, S.G. 1999. Patterning of the r7 and r8 photoreceptor cells of *Drosophila*: Evidence for induced and default cell-fate specification. *Development* **126:** 607–616.

Desalomon, C.H. and Spatz, H.C. 1983. Color-vision in *Drosophila melanogaster*—Wavelength discrimination. *J. Comp. Physiol.* **150:** 31–37.

Dill, M. and Heisenberg, M. 1995. Visual pattern memory without shape recognition. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **349:** 143–152.

Dill, M., Wolf, R., and Heisenberg, M. 1993. Visual pattern recognition in *Drosophila* involves retinotopic matching. *Nature* **365:** 751–753.

———. 1995. Behavioral analysis of *Drosophila* landmark learning in the flight simulator. *Learn. Mem.* **2:** 152–160.

Ernst, R. and Heisenberg, M. 1999. The memory template in *Drosophila* pattern vision at the flight simulator. *Vision Res.* **39:** 3920–3933.

Feiler, R., Harris, W.A., Kirschfeld, K., Wehrhahn, C., and Zuker, C.S. 1988. Targeted misexpression of a *Drosophila* opsin gene leads to altered visual function. *Nature* **333:** 737–741.

Feiler, R., Bjornson, R., Kirschfeld, K., Mismer, D., Rubin, G.M., Smith, D.P., Socolich, M., and Zuker, C.S. 1992. Ectopic expression of ultraviolet-rhodopsins in the blue photoreceptor cells of *Drosophila*: Visual physiology and photochemistry of transgenic animals. *J. Neurosci.* **12:** 3862–3868.

Fukushi, T. 1985. Visual learning in walking blowflies, *lucilia cuprina*. *J. Comp. Physiol. [A]* **157:** 771–778.

———. 1989. Learning and discrimination of coloured papers in the walking blowfly, *lucilia cuprina*. *J. Comp. Physiol. [A]* **166:** 57–64.

Gegenfurtner, K.R. and Kiper, D.C. 2003. Color vision. *Annu. Rev. Neurosci.* **26:** 181–206.

Gong, Z., Xia, S., Liu, L., Feng, C., and Guo, A. 1998. Operant visual learning and memory in *Drosophila* mutants dunce, amnesiac and radish. *J. Insect Physiol.* **44:** 1149–1158.

Götz, K.G. 1964. Optomotorische Untersuchung des Visuellen Systems einiger Augenmutanten der Fruchtfliege *Drosophila*. *Kybernetik* **2:** 77–92.

Greenspan, R.J. and van Swinderen, B. 2004. Cognitive consonance: Complex brain functions in the fruit fly and its relatives. *Trends Neurosci.* **27:** 707–711.

Guo, A. and Götz, K.G. 1997. Association of visual objects and olfactory cues in *Drosophila*. *Learn. Mem.* **4:** 192–204.

Guo, J. and Guo, A. 2005. Crossmodal interactions between olfactory and visual learning in *Drosophila*. *Science* **309:** 307–310.

Guo, A., Liu, L., Xia, S.-Z., Feng, C.-H., Wolf, R., and Heisenberg, M. 1996. Conditioned visual flight orientation in *Drosophila*; dependence on age, practice, and diet. *Learn. Mem.* **3:** 49–59.

Hardie, R.C. 1986. The photoreceptor array of the dipteran retina. *Trends Neurosci.* **9:** 419–423.

Heisenberg, M. 1995. Pattern recognition in insects. *Curr. Opin. Neurobiol.* **5:** 475–481.

Heisenberg, M. and Buchner, E. 1977. Role of retinula cell-types in visual behavior of *Drosophila melanogaster*. *J. Comp. Physiol.* **117:** 127–162.

Heisenberg, M. and Wolf, R. 1984. Vision in *Drosophila*. Genetics of microbehavior. Springer, Berlin, Heidelberg, New York, Tokyo.

Heisenberg, M., Wolf, R., and Brembs, B. 2001. Flexibility in a single behavioral variable of *Drosophila*. *Learn. Mem.* **8:** 1–10.

Hempel de Ibarra, N., Vorobyev, M., Brandt, R., and Giurfa, M. 2000. Detection of bright and dim colours by honeybees. *J. Exp. Biol.* **203:** 3289–3298.

Hempel de Ibarra, N., Giurfa, M. and Vorobyev, M. 2002. Discrimination of coloured patterns by honeybees through chromatic and achromatic cues. *J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol.* **188**: 503-512.

Kelber, A. 2005. Alternative use of chromatic and achromatic cues in a hawkmoth. *Proc. Biol. Sci.* **272:** 2143–2147.

Kelber, A., Vorobyev, M., and Osorio, D. 2003. Animal colour vision—behavioural tests and physiological concepts. *Biol. Rev. Camb. Philos. Soc.* **78:** 81–118.

Lehrer, M. 1987. To be or not to be a colour-seeing bee. *Israel J. Entomol.* **21:** 51–76.

Liu, L., Wang, X., Xia, S.Z., Feng, C.H., and Guo, A. 1998. Conditioned visual flight orientation in *Drosophila melanogaster* abolished by benzaldehyde. *Pharmacol. Biochem. Behav.* **61:** 349–355.

Liu, L., Wolf, R., Ernst, R., and Heisenberg, M. 1999. Context generalization in *Drosophila* visual learning requires the mushroom bodies. *Nature* **400:** 753–756.

Livingstone, M. and Hubel, D. 1988. Segregation of form, color, movement, and depth: Anatomy, physiology, and perception. *Science* **240:** 740–749.

Menne, D. and Spatz, H.C. 1977. Color-vision in *Drosophila melanogaster*. *J. Comp. Physiol.* **114:** 301–312.

Osorio, D. and Vorobyev, M. 2005. Photoreceptor spectral sensitivities in terrestrial animals: Adaptations for luminance and colour vision. *Proc. Biol. Sci.* **272:** 1745–1752.

Quinn, W.G., Harris, W.A., and Benzer, S. 1974. Conditioned behavior in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci.* **71:** 708–712.

Salcedo, E., Zheng, L., Phistry, M., Bagg, E.E., and Britt, S.G. 2003. Molecular basis for ultraviolet vision in invertebrates. *J. Neurosci.* **23:** 10873–10878.

Spatz, H.C., Emanns, A., and Reichert, H. 1974. Associative learning of *Drosophila melanogaster*. *Nature* **248:** 359–361.

Stark, W.S. and Thomas, C.F. 2004. Microscopy of multiple visual receptor types in *Drosophila*. *Mol. Vis.* **10:** 943–955.

Stavenga, D.G., Smits, R.P., and Hoenders, B.J. 1993. Simple exponential functions describing the absorbance bands of visual pigment spectra. *Vision Res.* **33:** 1011–1017.

Tang, S. and Guo, A. 2001. Choice behavior of *Drosophila* facing contradictory visual cues. *Science* **294:** 1543–1547.

Tang, S.M., Wolf, R., Xu, S.P., and Heisenberg, M. 2004. Visual pattern recognition in *Drosophila* is invariant for retinal position. *Science* **305:** 1020–1022.

Troje, N. 1993. Spectral categories in the learning-behavior of blowflies. *Zeitschrift Fur Naturforschung C-a J. Biosci.* **48:** 96–104.

van Swinderen, B. and Greenspan, R.J. 2003. Salience modulates 20-30 hz brain activity in *Drosophila*. *Nat. Neurosci.* **6:** 579–586.

Vorobyev, M. and Osorio, D. 1998. Receptor noise as a determinant of

colour thresholds. *Proc. Biol. Sci.* **265:** 351–358.
Vorobyev, M., Osorio, D., Bennett, A.T., Marshall, N.J., and Cuthill, I.C. 1998. Tetrachromacy, oil droplets and bird plumage colours. *J. Comp. Physiol. [A]* **183:** 621–633.
Vorobyev, M., Brandt, R., Peitsch, D., Laughlin, S.B., and Menzel, R. 2001. Colour thresholds and receptor noise: Behaviour and physiology compared. *Vision Res.* **41:** 639–653.
Wang, X., Liu, L., Xia, S.Z., Feng, C.H., and Guo, A. 1998. Relationship between visual learning/memory ability and brain camp level in *Drosophila*. *Sci. China C Life Sci.* **41:** 503–511.
Wang, S., Li, Y., Feng, C., and Guo, A. 2003. Dissociation of visual associative and motor learning in *Drosophila* at the flight simulator. *Behav. Processes* **64:** 57–70.
Wolf, R. and Heisenberg, M. 1991. Basic organization of operant behavior as revealed in *Drosophila* flight orientation. *J. Comp. Physiol. [A]* **169:** 699–705.
———. 1995. Learning of *Drosophila* in the flight simulator: Classically conditioned visual pattern discrimination. In *Nervous systems and behaviour. Proceedings of the 4th International Congress of Neuroethology* (eds. M. Burrows et al.), p. 184. Georg Thieme Verlag, Stuttgart, New York.
———. 1997. Visual space from visual motion: Turn integration in tethered flying *Drosophila*. *Learn. Mem.* **4:** 318–327.
———. 1998. *Fifth International Congress of Neuroethology,* San Diego, CA.
Wolf, R., Voss, A., Hein, S., and Heisenberg, M. 1992. Can a fly ride a bicycle? Discussion on natural and artificial low-level seeing systems. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* **337:** 261–269.
Wolf, R., Wittig, T., Liu, L., Wustmann, G., Eyding, D., and Heisenberg, M. 1998. *Drosophila* mushroom bodies are dispensable for visual, tactile and motor learning. *Learn. Mem.* **5:** 166–178.
Wyszecki, G. and Stiles, W.S. 1982. *Color science—concepts and methods, quantitative data and formulae*. Wiley, New York.
Xia, S.Z., Liu, L., Feng, C.H., and Guo, A. 1997a. Memory consolidation in *Drosophila* operant visual learning. *Learn. Mem.* **4:** 205–218.
———. 1997b. Nutritional effects on operant visual learning in *Drosophila melanogaster*. *Physiol. Behav.* **62:** 263–271.
Xia, S.Z., Feng, C.H., and Guo, A.K. 1999. Temporary amnesia induced by cold anesthesia and hypoxia in *Drosophila*. *Physiol. Behav.* **65:** 617–623.

Received February 27, 2006; accepted in revised form June 20, 2006.

OPEN ACCESS Freely available online

PLoS one

Order in Spontaneous Behavior

Alexander Maye[1], Chih-hao Hsieh[2], George Sugihara[2], Björn Brembs[3]*

1 Universitätsklinikum Hamburg-Eppendorf, Zentrum für Experimentelle Medizin, Institut für Neurophysiologie und Pathophysiologie, Hamburg, Germany, **2** Scripps Institution of Oceanography, University of California San Diego, La Jolla, California, United States of America, **3** Freie Universität Berlin, Institut für Biologie–Neurobiologie, Berlin, Germany

Brains are usually described as input/output systems: they transform sensory input into motor output. However, the motor output of brains (behavior) is notoriously variable, even under identical sensory conditions. The question of whether this behavioral variability merely reflects residual deviations due to extrinsic random noise in such otherwise deterministic systems or an intrinsic, adaptive indeterminacy trait is central for the basic understanding of brain function. Instead of random noise, we find a fractal order (resembling Lévy flights) in the temporal structure of spontaneous flight maneuvers in tethered *Drosophila* fruit flies. Lévy-like probabilistic behavior patterns are evolutionarily conserved, suggesting a general neural mechanism underlying spontaneous behavior. *Drosophila* can produce these patterns endogenously, without any external cues. The fly's behavior is controlled by brain circuits which operate as a nonlinear system with unstable dynamics far from equilibrium. These findings suggest that both general models of brain function and autonomous agents ought to include biologically relevant nonlinear, endogenous behavior-initiating mechanisms if they strive to realistically simulate biological brains or out-compete other agents.

Citation: Maye A, Hsieh C-h, Sugihara G, Brembs B (2007) Order in Spontaneous Behavior. PLoS ONE 2(5): e443. doi:10.1371/journal.pone.0000443

INTRODUCTION

According to Laplace, randomness is only a measure of our "ignorance of the different causes involved in the production of events." [1] Probably the most fundamental feature of modern scientific inquiry is the ability to find these causes and predict future events [1,2]. Reflecting this view, animals are thought to operate according to laws firmly tying behavioral 'responses' to environmental variables: "[N]euroscience, over the last 30 years, […] each year brings a greater understanding of the mechanical way with which we perceive, we remember, we speak, we feel." [3] Once these laws are known, the behavior of any animal at any time can be predicted from the current environmental situation [4]: "We cannot prove […] that human behavior […] is fully determined, but the position becomes more plausible as facts accumulate." [5] This does not necessarily imply that the same stimulus always elicits the same behavior, but that each behavior is a response to a stimulus: "Indeed, so pervasive is the basic assumption of this model that it is common to refer to any behaviour as a 'response' and thus by implication […] assume that there must be an eliciting stimulus." [6] This basic tenet not only guides basic neurobiological and psychological research but has been the foundation for a great many robotics applications [7–9] as well as for speculations on the future societal impact of neuroscience [3,10,11]. Basically, the brain is seen an input/output device: "brain function is ultimately best understood in terms of input/output transformations and how they are produced" [12]. Contending that less complex brains would be more amenable to this research, the study of invertebrate and in particular fly behavior developed into a prominent focus of attention [7,8,13,14].

However, even the best-understood behavioral systems display a residual of variability, which has so far prevented exact predictability of individual behavior. There are a number of systems from single neurons and synapses [15,16] to invertebrate [17,18] and vertebrate animals including humans [19–21], which even generate variable output despite no variations in input at all, leading to difficulties reproducing even tightly controlled experiments [22]. This variability is often classified as random noise, a by-product of a complex brain [23,24]. Documented sources of noise range from genetic and historical variations [23] to neural noise [24,25] or stochastic fluctuations in macromolecule number [26]. This noise requires compensatory homeostatic mechanisms to ensure stable neuronal and network function over extended periods of time [27]. Because of the obvious analogy, we term the hypothesis that brains are deterministic input/output systems with added noise the 'robot-hypothesis' (Fig. 1a). A less prominent alternative explanation contends that some of the variability is adaptive and irreducible [19,20,28]. According to this latter view, individual behavior is fundamentally indeterministic (not fundamentally deterministic but noisy) and precise prediction principally (not only technically) impossible (Fig. 1b). It is critical to emphasize at this point that the processes leading to behavioral indeterminacy may very well be deterministic: indeterministic output of deterministic systems is a well-known phenomenon [29].

Analyzing the structure of behavioral variability may provide evidence for understanding whether the variability is the result of cumulated errors in an imperfectly wired brain (system noise) or whether the variability is under neural control. In this study, we take advantage of turning behavior in tethered *Drosophila*; this system provides superb control over the perceived environment for a true assessment of the spontaneity of the behavior, while at the same time offering easily quantifiable behavioral dynamics (Fig. 2). Most importantly, we eliminate any potential nonlinear effects which could arise from a closed reafferent feedback loop between the animal's behavior and its environment by opening this loop to

Academic Editor: Martin Giurfa, Centre de Recherches su la Cognition Animale-Centre National de la Recherche Scientifique and Université Paul Sabatier, France

Received October 23, 2006; **Accepted** April 18, 2007; **Published** May 16, 2007

Funding: Supported by the DFG (BR 1893/3-2)

Competing Interests: The authors have declared that no competing interests exist.

* **To whom correspondence should be addressed.** E-mail: bjoern@brembs.net

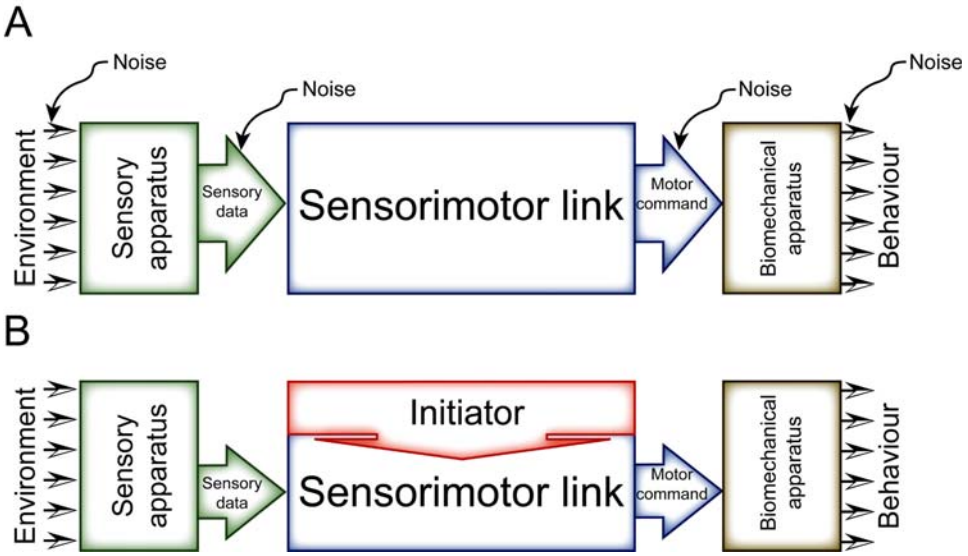


Figure 1. Alternative models conceptualizing the open-loop experiment. A–According to the robot-hypothesis, there is an unambiguous mapping of sensory input to behavioral output. If the behavioral output is not constant in a constant environment, there are a number of possible sources of noise, which would be responsible for the varying output. B–In a competing hypothesis, non-constant output is generated intrinsically by an initiator of behavioral activity. Note that the sources of noise have been omitted in B merely because their contribution may be small, compared to that of the initiator, not because they are thought to be non-existent.
doi:10.1371/journal.pone.0000443.g001

study intrinsically generated behavior, without any environmental feedback. Thus, the environment is kept so constant (both between and within experiments), that any remaining minute variation in it must be infinitely smaller than any of the stimuli known to trigger turning behavior [30]. Moreover, the temporal distribution of any such remaining environmental fluctuations can be assumed to be Gaussian. We know of no other intact preparation affording such minute control. We chose the temporal sequence of highly stereotyped flight maneuvers producing short bursts of yaw-torque ('torque spikes'; corresponding to body-saccades in free flight [31]) for our analysis, because they have been repeatedly both classified as single units of behavior and used for quantitative behavioral analysis. Tethered *Drosophila* produce these spikes in a probabilistic manner not only in response to visual stimulation [14], but also if the stimulus situation is constant [30] (see also Figs. S1 and S2). Freely flying flies do not offer this distinction, as one cannot discern spontaneous body-saccades from elicited body-saccades [32].

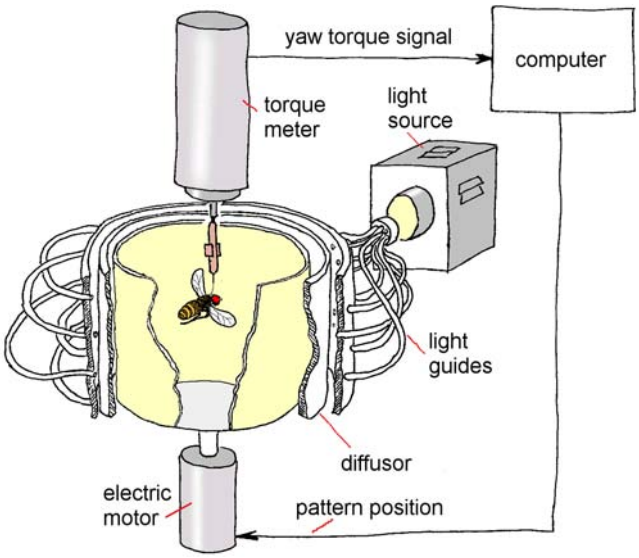


Figure 2. Flight simulator set-up. The fly is flying stationarily in a cylindrical arena homogeneously illuminated from behind. The fly's tendency to perform left or right turns (yaw torque) is measured continuously and fed into the computer. In closed-loop, the computer controls arena rotation (single stripe or uniform texture as patterns on the arena wall). An additional white screen (not shown) covered the arena from above for all groups.
doi:10.1371/journal.pone.0000443.g002

RESULTS

Spontaneous behavior is not simply random

Naively, if the production of torque spikes in our featureless or uniform environment were due to random noise in the *Drosophila* brain or from any uncontrollable input, the time intervals between spikes (inter-spike interval, ISI) should reflect this stochasticity, much like the hiss of static from a radio between stations. Given a certain mean spike rate, the most straightforward assumption is to expect a stochastic procedure to behave according to a Poisson process [24,25,33]. In other words, this situation should represent a natural system for generating random numbers. Therefore, we adapted a recently developed computational method, Geometric Random Inner Products (GRIP) [34], to quantify the randomness of the ISI sequences of three groups of flies. The first group ('*openloop*') flew in a completely featureless white panorama (i.e., without any feedback from the uniform environment–open loop). The ISI sequence in these flies must be generated entirely spontaneously. The second group ('*onestripe*') flew in an environment that contained a single black stripe as a visual landmark (pattern) in a flight simulator situation that allowed for straight flight in optomotor balance (i.e. the fly could use its yaw torque to control the angular position of the stripe–closed loop). Flies from this group not only received reafferent feedback from the effects their maneuvers had on the angular position of the stripe, but it is

also known that such stripes elicit optomotor and fixation responses [35] (see also Fig. S2), providing for an input/output control group. The third group ('*uniform*') flew in a uniformly textured environment that was otherwise free of any singularities (i.e., closed loop, the fly could use its yaw torque to control the angular position of the evenly dashed environment). This arrangement also allows for straight flight in optomotor balance but it does not elicit any fixation or directional preferences as the *onestripe* situation. Therefore the *uniform* group constitutes an intermediate case. A significant deviation from ideal randomness in any of these groups would contradict the 'robot-hypothesis'. GRIP results show that fly behavior deviates from perfect randomness (Fig. 3a). In all our groups, this deviation even exceeds the values from a computer-generated Poisson process (Kruskal-Wallis ANOVA: $H(3, N = 52) = 17.2$; $p<0.0007$. In post-hoc tests, all fly values were significantly higher than the *poisson* control values, $p<0.03$ in all cases). Plotting the number of ISIs as a function of ISI duration reveals an overrepresentation of long ISIs with respect to an exponential distribution (so-called heavy-tailed distributions; see Fig. S3). Thus, the simplest hypothesis that first-order noise underlies variable spike generation in a constant environment has to be rejected.

One may argue that the assumption of a constant spike rate is arbitrary, overly simplistic and that more complex stochastic processes are likely to be at work, even in flies. A well-known example of such stochastic processes is a doubly stochastic Poisson process (or Cox Process) [36,37]. A Cox process is essentially a Poisson process in which the rate is not constant, but fluctuates randomly. In our example, a fly's spike rate may change in response to uncontrolled, random events in the fly's environment or to random events within the fly. Cox processes can generate heavy-tailed distributions, sometimes also called power-law distributions. Power laws are among the most frequent scaling laws that describe the scale invariance found in many natural phenomena and can be seen as a straight line on a log-log graph of the data. Therefore, we plotted the number of ISIs as a function of ISI duration on a double logarithmic scale. To simulate a Cox process, we used the instantaneous spike rates from the flies in the *openloop* group to drive the rate of a Poisson process (*cox*; see *Methods* for details). A very similar process has previously been used to successfully model the spike trains of neurons such as those in the cat visual cortex [38]. We found inverse power-law distributions both in the timing of fly ISIs and in the *cox* group (Fig. 3b). For the two fly groups without a singularity in the environment (*openloop* and *uniform*) and for the Cox process, the duration of ISIs decayed according to a non-Gaussian Lévy distribution (with the Lévy exponent $1<\mu<3$). Conspicuously, the Cox process is also Lévy distributed. Do such results provide any leads for investigating the potential mechanisms underlying spontaneous turning behavior?

Lévy flights, a special class of Markov processes, are scale invariant and often associated with power-laws described in many other systems [39–41]. A Lévy flight can be conceptualized as a process which first chooses a direction at random and then keeps flying for a distance drawn at random from a Lévy distribution [42]. The Cox process, although not working in this way, still yields a Lévy distribution. It has also been proposed that systems with a large number of nonlinearly coupled subsystems also may exhibit Lévy distributions [43,44]. Clearly, "the presence of such distributions tells us nothing about the mechanisms that give rise to them" [45]. Notwithstanding, all the more common stochastic processes which can give rise to Lévy distributions imply second-order (or conditional) stochastics. These processes share the property that the conditional probability distribution of the next step depends only on their current state and not on the steps in the past (i.e., no memory). The Cox process is a classic representative of this class of conditional stochastic processes.

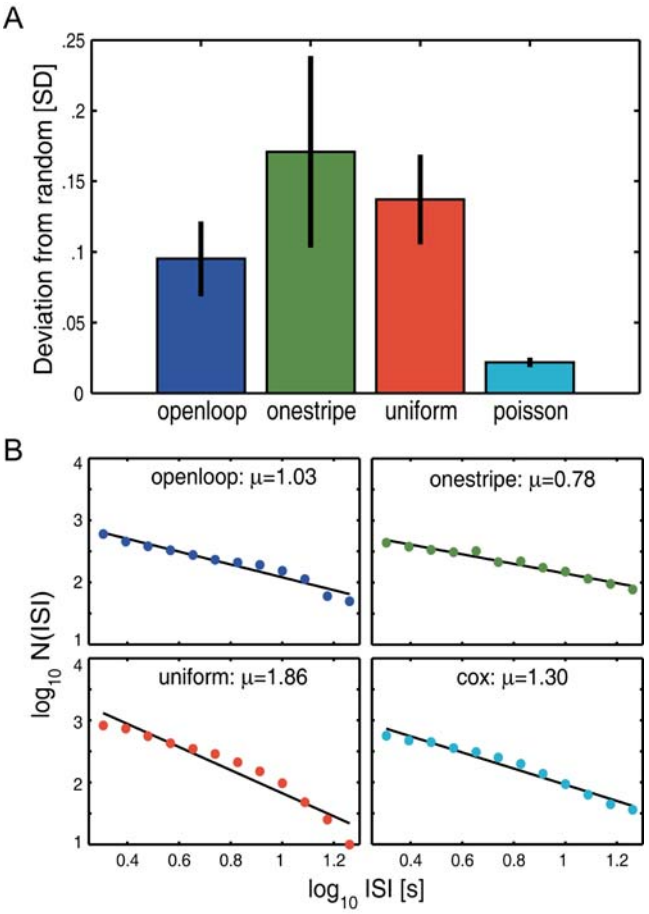


Figure 3. Spontaneous behavior is not simply random. A–GRIP analysis of ISIs. Plotted are the mean standard deviations from the theoretically expected random value for fly ISI series and the random series generated by a Poisson process. The fly deviations are all significantly larger than the values for the computer-generated series. B–Log-log plots of ISIs. The Lévy exponent μ is calculated from the inclination of the linear fit. A Lévy distribution is defined as $1<\mu<3$. Smaller values indicate a larger proportion of long ISIs. A Cox Process (*cox*) reveals a similar power-law structure as the flies. Error bars are S.E.M.s throughout. See *Methods* for details and statistics.
doi:10.1371/journal.pone.0000443.g003

Spontaneous behavior reveals a fractal order

A standard method of testing for renewal processes without memory (i.e., Markov, Lévy or Cox processes) is to compare the original sequence to randomly shuffled ("surrogate") sequences. This surrogate data set maintains the same relative frequency of ISI durations as the original data, but destroys the ordering of the intervals. A significant difference between surrogate data and original data indicates that conditional probabilities are not involved in the generation of the series. For this comparison, we first computed the correlation dimension [46] for the original ISI series which yields a sequence-dependent measure for each fly. The correlation dimension is a measure of the dimensionality of the space occupied by a particular ISI sequence (similar to the less reliable *fractal dimension*). If the correlation dimension converges on a fractional value, the ISI sequence is termed 'fractal'. This first step of computing individual correlation dimensions already hints

at a difference between the stochastic ISI series and the fly series: all four traces appear very similar, but the fly data each converge on a specific dimension while the *cox* series diverges with increasing embedding dimensionality (Fig. 4a). The convergence of the correlation dimensions for fly data suggests a fractal order in the fly ISI series and not in the *cox* series. However, these differences are rather subtle and somewhat subjective. In the decisive second step, we calculated the probability that any randomly shuffled sequence of ISIs could have produced the same outcome. The results show that most likely the recorded sequence of ISIs–and not any random shuffling thereof–is responsible for the computed correlation dimensions, rejecting the hypothesis of second-order stochastics dominating the generation of spontaneous turning behavior in *Drosophila* (Fig. 4b). Similar to sequences of ISIs recorded in the monkey basal ganglia [47], sequences of fly ISIs are not entirely defined by their probability distribution. In contrast, we can not reject the hypothesis that any sequence could generate the computed correlation dimension for the *cox* series, at the .05 criterion. A Kruskal-Wallis ANOVA was significant for the shuffled correlation dimension probabilities: $H(3, N = 52) = 24.7$; $p<0.0001$. All fly probabilities were significantly lower than the *cox* probability ($p<0.02$ in all cases). This outcome rules out renewal processes as the main mechanism generating spontaneous turns in *Drosophila*. Specifically, this excludes Cox processes or other superpositions of random processes, which one could assume if several separate processes in the brain lead to torque spike production or for the superposition of environmentally and endogenously triggered torque spikes.

Long-range correlations in the behavior imply nonlinearity

However, there are yet more complex composite stochastic models which, like the fly data, can exhibit a fractal structure [15,48]. These models combine a multitude of stochastic processes by deterministic rules. For instance, the so-called "branched Poisson process" (*BPP*, see Fig. S4a) consists of a cascade of Poisson processes each driving the rate of the next via a filter function [48]. The combined output of all these processes constitutes the output of the entire *BPP*. Such processes can produce ISI series which do show fractal characteristics and their probability of shuffled data to yield the same correlation dimension comes to lie in-between standard stochastics and fly data, such that they cannot easily be distinguished from either of the two (data not shown). The results from surrogate data imply a form of memory in both spontaneous flight behavior and to a certain degree also in *BPP*s that lasts beyond the current time point. Specific ISI durations are determined in part by the timing of other spike(s), and ISI durations fluctuate over time rather than relaxing to a homeostatic steady state. Such a memory can lead to long-range correlations in the data which may be the reason why the shuffled data fail to reproduce the original correlation dimension. A sensitive method to detect these correlations is to calculate the root mean square (r.m.s.) fluctuations in the ISI series (see *Methods*). For uncorrelated time series r.m.s. fluctuations decay according to a power-law with an exponent α of ½. If the exponent deviates from ½, long-range correlations exist in the time series [32,49]. This computation shows significant deviations from ½ for all the fly series (Fig. 5; t-test against single value: $p<0.001$ for all three groups). Besides the fly data, we tested two forms of BPP, one with a linear filter function and one with a nonlinear filter. We found that the presence of long-range correlations was dependent on the nonlinearity of the filter function (Fig. 5; t-test against single value: $p<0.3$ for *BPP* with linear filter and $p<0.04$ for *BPP* with nonlinear filter). However, the value for the *BPP* with the nonlinear filter function is still significantly smaller than the value for the *openloop* group, to which it was fitted (Mann-Whitney U-Test, $p<0.005$), ruling out even *BPP*s with nonlinear filters as an appropriate model for spontaneous flight behavior in *Drosophila*.

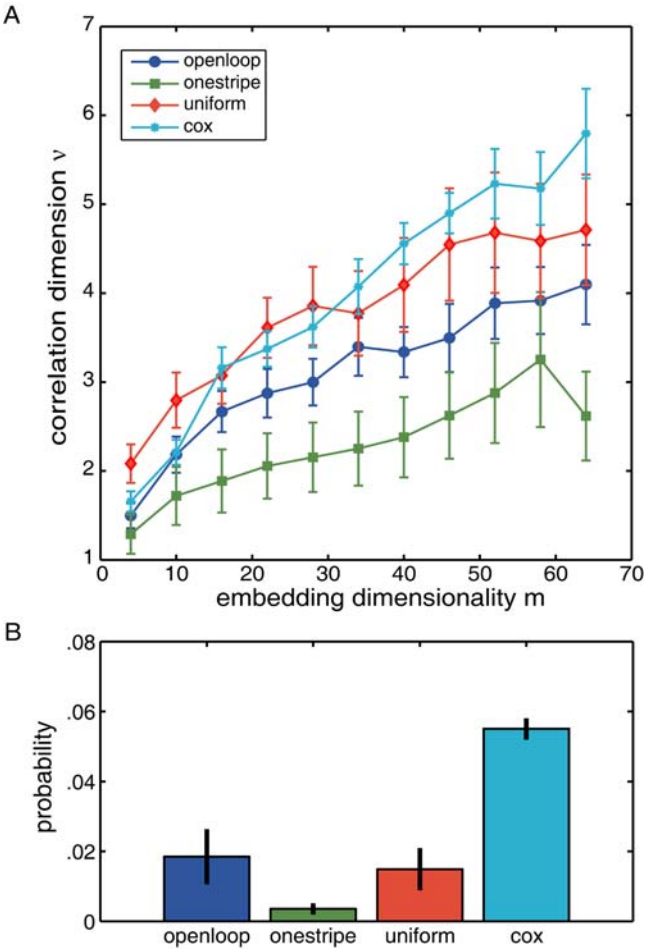


Figure 4. Correlation dimension. A–While the correlation dimension converges on a group-specific value with increasing embedding dimension for fly-generated ISIs (*openloop, onestripe, uniform*), a number sequence generated randomly by a Cox Process (*cox*) diverges. B–Probability to obtain the computed correlation dimensions in A by random shuffling of the original data. While the *cox* group exceeds an alpha value of .05, the three fly groups stay well below that threshold.
doi:10.1371/journal.pone.0000443.g004

The dependence of the α-values on the nonlinearity contained in the BPPs entices to hypothesize that what is needed to achieve long-term correlations such as those observed in flies (this study and [32]) and other animals such as albatrosses [49] are not essentially random processes connected by nonlinear mechanisms, but rather essentially nonlinear processes containing random noise. We thus employed a recently developed method which distinguishes essentially stochastic from essentially nonlinear time series.

Nonlinearity in the behavior implies instability in the brain

All the previous analyses showed that *Drosophila* turning behavior is at least partially non-random. Information theory tells us that in this case the ISI series contain some sort of information [50].

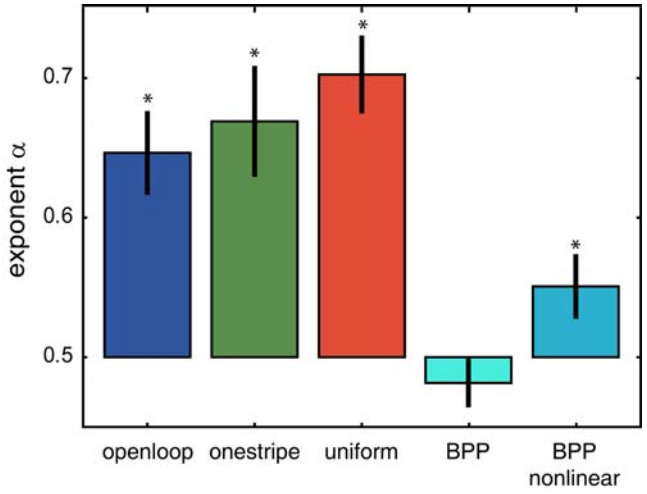


Figure 5. Long-range correlations in fly ISIs. If the slope of the log-log plots of the r.m.s. fluctuation (exponent α, see *Methods*) deviates significantly from ½, long-range correlations exist in the time series. All three fly groups show a significant deviation from 0.5. The deviation of branched Poisson processes (*BPP*), however, depends on the non-linearity of the filter function used to drive the Poisson processes and is significantly smaller than that of fly ISI series. *-significant difference from 0.5.
doi:10.1371/journal.pone.0000443.g005

Forecasting analyses can use this information to predict parts of the sequences. Similar to a weather forecast, forecasting analyses use part of the time series to derive a mathematical model which predicts the remainder of the series. The computed prediction is then compared to the actual series to obtain a correlation coefficient which is a measure for the accuracy of the prediction. Specifically, nonlinear forecasting comprises a set of established methods from nonlinear time series analysis that involve state space reconstruction with lagged coordinate embeddings [51,52]. These methods take advantage of the loss of information in nonlinear time series to distinguish them from essentially stochastic (high-dimensional, linear) series. In a two-step procedure, we use the Simplex-projection [52] to identify the best embedding dimension and the S-map procedures [53] to assess the non-linearity of the data (Fig. 6). The method of S-maps relies on fitting a series of models (from linear to nonlinear) where the degree of nonlinearity is controlled by a local weighting parameter Θ. Improved out-of-sample forecast skill with increasingly nonlinear models (larger Θ) indicates that the underlying dynamics were themselves nonlinear [53]. The fly ISI time series show a weak but consistent improved forecast skill with increasing Θ, exhibiting a nonlinear signature (Fig. 6a). However, the overall nonlinear forecast skill is rather low for fly ISI series. To exclude any loss of information introduced by spike detection, we also evaluated the raw yaw torque data series. Analyzing the raw data with the two-step S-Map method also yields increased forecast skill for increasingly nonlinear models, this time with a profoundly larger overall forecast skill (Fig. 6a). This result excludes all essentially stochastic models irrespective of their memory as the basis for fly turning behavior and firmly establishes nonlinearity as the main mechanism.

A popular concept of animal behaviour includes the transition between motivational states. True state shifts are not random features of a time series but instead formally associated with the idea of nonlinearity [54]. Hallmarks of state shifts are e.g. alternative basins of attraction, multiple stable states, hysteresis and fold catastrophe, all of which require the underlying dynamics to be nonlinear in origin [53]. Our analysis suggests that the brain structures generating yaw-torque spikes also operate according to nonlinear rules, similar to the ones discovered in many other natural systems. Nonlinearity is ubiquitous in nervous systems, from single neurons to circuits [29]. A critic may thus argue that the nonlinear signature we find in the fly behavior is merely a reflection of this already well-known property and not indicative of fine-tuned neural control systems. To test this hypothesis, we adapted a virtual agent (i.e., a computer model or *automat*) [55] consisting of three coupled nonlinear generators for comparison with our fly raw data. The agent is intuitively very appealing on a number of levels. First, its structure resembles one which may be expected for fly torque production: one of the generators (the "activator") activates the other two ("left torque" and "right torque"), which resembles how a motor command from the brain would activate motor patterns in the thoracic ganglion. The two torque generators mutually inhibit each other, preventing the simultaneous activation of right and left turns (Fig. S4b). Second, the original agent's search behavior is similar to a Lévy walk [55]. Third, the *automat* can be tuned so that its open-loop output shows a similar nonlinear signature as fly turning behavior (Fig. 6a, "automat 1"). Fourth, the *automat* can be adjusted such that its output appears to be qualitatively similar to fly open-loop turning behavior (Fig. 6b, "automat 2"). Thus, it seems that indeed the biologically plausible, nonlinear processes in the agent are sufficient to model fly behavior. However, interestingly, if the *automat* is tuned to resemble fly behavior, it does not reveal a nonlinear signature in the S-Map procedure (Fig. 6a, "automat 2"). Indeed, to reveal its nonlinear signature, the *automat* has to be adjusted such that the nonlinear generators operate under unstable conditions, at which point the output fails to resemble fly behavior (Fig. 6b, "automat 1"). This experiment falsifies the initial hypothesis that the nonlinear signature we find in fly behavior is merely a reflection of the well-known nonlinear properties of brains. Nonlinearity is a necessary, but not a sufficient criterion: only if the systems operate under unstable conditions does the output reveal significant nonlinearity (see Fig. S5 for additional S-Map results). The failure of this agent to adequately model fly behavior is an example for the rarely appreciated property of nonlinear systems to produce linear output under equilibrium conditions.

DISCUSSION

Even small fly brains can control behavior with minute precision. For instance, male house flies closely track the evading flight maneuvers of female flies with only a lag of about 30ms [56]. Input/output models reproduce these chasing flights with high fidelity [56–58]. Such input/output systems provide the flies with exquisite control over their turning maneuvers. Nevertheless, bereft of visual input flies produce turning maneuvers, the variability of which would never allow them to stay clear of obstacles, land on food, let alone catch the mate. Where does this variability come from? How does the female fly produce seemingly random turn maneuvers, making it so difficult for the male fly to follow? Obviously, the amount of behavioral variability is in itself variable and must be under the control of the brain. How does the brain do this?

Behavioral variability is a well-known phenomenon. It is so pervasive that the semi-serious Harvard Law of Animal Behavior was coined: "Under carefully controlled experimental circumstances, an animal will behave as it damned well pleases." It is the source of this variability which is under scrutiny here. The current neuroscientific consensus posits that the source of the variability is

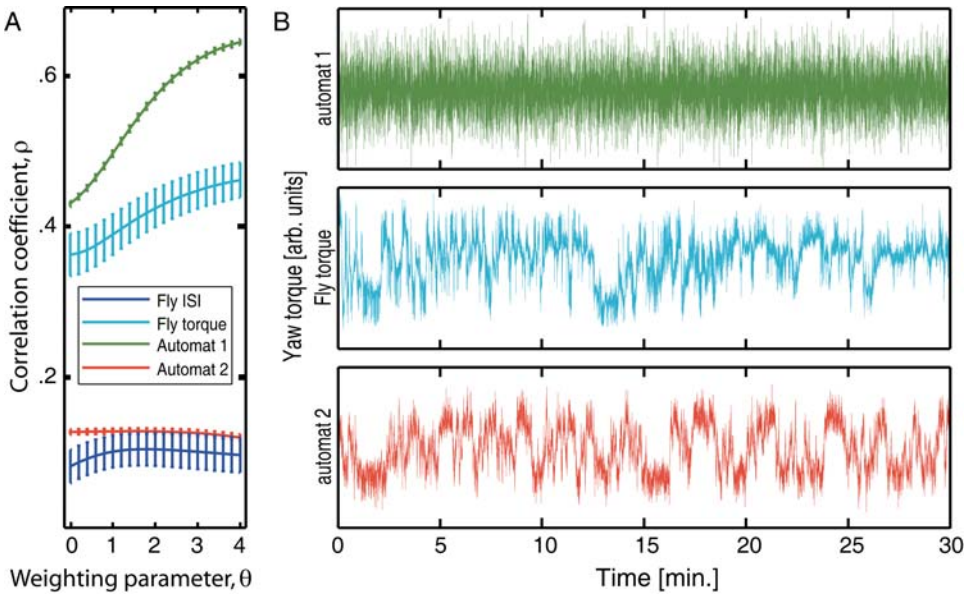


Figure 6. Nonlinearity implies instability. A–S-Map results. Depicted are the averaged results for fly ISIs and raw yaw torque series (for clarity, only *openloop* data are shown here), together with two automat simulations. The fly ISI series shows a slightly improved forecast skill with increasingly nonlinear S-map solutions (increasing Θ). Fly yaw torque series yield both a better overall forecast skill as well as increased nonlinear improvement. The automat simulation can be tuned to produce both linear and nonlinear output. B–Sample raw yaw torque data traces from a real fly and the two versions of the simulated agent depicted in A (automat 1, automat 2). S-Map results for the other two groups are depicted in Fig. S5.
doi:10.1371/journal.pone.0000443.g006

noise, rendering the variability random or stochastic. We show here that random noise cannot be the sole source of behavioral variability. In addition to the inevitable noise component, we detected a nonlinear signature suggesting deterministic endogenous processes (i.e., an initiator) involved in generating behavioral variability. It is this combination of chance and necessity that renders individual behavior so notoriously unpredictable. The consequences of this result are profound and may seem contradictory at first: despite being largely deterministic, this initiator falsifies the notion of behavioral determinism. By virtue of its sensitivity to initial conditions, the initiator renders genuine spontaneity ("voluntariness" [30]) a biological trait even in flies.

Even fly brains are more than just input/output systems

The variability in spontaneous fly turning behavior is not solely due to nonlinearity; rather, the nonlinear processes controlling the behavior also have to operate at just the right parameters to produce instability. Moreover, the number of these nonlinear processes has to be small, as nonlinear signatures disappear with increasing superposition of multiple nonlinear processes [59,60]. Thus, flies are more than simple input/output machines. Similar to flies, human brains also are notorious for their variability and even devote most of their energy budget to intrinsic processing [21]. Our study supports the hypothesis that the nonlinear processes underlying spontaneous behavior initiation have evolved to generate behavioral indeterminacy: The choice of what behavior to produce in the next moment is rarely determinable exactly, but only probabilistically [17,19,20]. Implicitly, game theory, the biological study of choice behavior and neuroeconomics have incorporated this feature on an empirical basis [61–65]. If our results from a small fly brain hold also for more complex brains, they suggest that the biological basis of the widespread phenomenon of behavioral indeterminacy can be investigated. For instance, inhibiting neurons forming the ellipsoid body, a neuropil structure in the fly central brain, shifts the temporal structure of *Drosophila* walking behavior from non-Gaussian to Gaussian [41]. It will be interesting to screen for the neurons involved in initiating spontaneous turning behavior as well. Classes of behaviors may be controlled by separate initiators. For instance, human eye saccades show a Gaussian temporal structure [66], whereas communication and travel are clearly non-Gaussian [33,67,68]. Also in humans, a "default network" seems to be responsible for spontaneous, stimulus-independent thought [69]. Our data may help explain the notorious difficulty to exactly reproduce behavioral results even when they are under extremely tight experimental control [22]. We hypothesize that the degree to which an animal behaves deterministically is shaped by evolution and thus depends on the ecological niche for which the behavior evolved.

Optimal searching behavior

What, if any, ecological niche has spontaneous flight behavior in *Drosophila* evolved for? Given the artificial circumstances of our experiments, one would assume that the flies were highly motivated to find an escape. Could the heavy-tailed distribution of turning maneuvers constitute an evolved search behavior? A number of publications have reported Lévy-like search strategies in analyses of a variety of behaviors from plankton to humans [32,33,49,68,70]. Lévy flights or walks cause the organism to hit a fractal clustered set of points. Surprisingly, flies can in principle produce such behavioral patterns even without any environmental feedback at all (*openloop*, Fig. 3b). One would conclude that internal timing rather than external cues is organizing this behavior. Obviously, environmental feedback can alter the timing of the torque spikes and can thus increase (*uniform*) or decrease (*onestripe*) the distribution characteristics (Fig. 3b). In our setup, the flies can only receive horizontal visual feedback. Nevertheless, the *uniform* group already shows a Lévy exponent very close to the $\mu \approx 2$ which was observed in freely flying *Drosophila* [32]. Movement patterns with such properties are known to constitute a mathematically optimal search strategy for randomly and sparsely distributed

resources [39]. Thus, it appears that all that is required to produce such an optimal search strategy is a default network which spontaneously generates behavior that is already close to optimal, combined with very rudimentary environmental feedback to adjust the default state to the environment at hand. It seems that one component of such a default strategy in *Drosophila* are search spirals, which arise when multiple body-saccades in the same direction are generated with only short ISIs [32] (see also Fig. S2). Conventional experiments with freely moving animals could never have shown this simple relationship. Indeed, in free flight, changes in environmental feedback did not significantly alter the search characteristics [32]. The discovery of near-optimal built-in search strategies enables us now to investigate the brain mechanisms behind optimal foraging in a genetically tractable model organism. Interestingly, these strategies are not random but nevertheless indeterminate.

New models of brain function

Because theoretical work suggests a range of competitive advantages for indeterminate behavior in virtually all animals [19,61–65,71], the structure of the indeterminacy should be incorporated explicitly into models of general brain function and autonomous agents. What would such future models of brain (or agent) function look like? Nonlinear models displaying probabilistic behavior patterns can in principle be fairly simple [55]. The nonlinear mechanisms need still to be influenced by the environment both in a feed-forward form (the sensorimotor link) [7,13,14,72] and by reafferent feedback control (Fig. 7) [73,74]. Our data raise the suspicion that future models of the brain may have to implement this or a related component for spontaneous behavior initiation, if they strive to be biologically realistic, outcompeting other models/agents. Recently, a new class of agents was introduced, which incorporated some of these ideas [75].

What is the advantage of nonlinear over random?

But what, if any, difference does it make when behavioral variability–despite being largely unpredictable–is not entirely stochastic, but nonlinear and unstable? The tedious distinction between random noise and unstable nonlinearity is worthwhile, because the former points to extrinsic origins of variability, whereas the latter indicates intrinsic origins. Technical advances frequently lead to a significant increase in signal to noise ratios. Such advances would increase the predictability of a brain where the main source of variability stems from noise. In contrast, noise reductions will only marginally change the predictability of a nonlinear brain whose output is fundamentally indeterministic, despite the deterministic rules that govern it. Given that there is a cost associated with producing indeterminate behavior [61], it is a straightforward inference that these latter rules have evolved specifically to generate varying degrees of behavioral indeterminism [23], as exemplified above in the case of the chasing house flies.

Brains are simultaneously indeterministic and deterministic for a reason

This insight has implications for our understanding of the general function of brains. The most fundamental brain function is to produce adaptive behavior. Adaptive behavior is the ability to orient toward specific goals in the environment and to control actions flexibly in pursuit of those goals. By and large, the everyday world we live in is Newtonian: predictable and deterministic. If we lose balance, we fall, if we neglect obstacles in our path, we collide with them and if we reach for an object, we can grasp it. Hence, no ambulatory animal could survive without its set of adaptive, hard-wired sensorimotor rules shaped by evolution and tuned by experience. No male house fly would ever catch its mate. At the same time, the world is full of surprises: the unexpected pursuit by a male house fly, the rejection of your manuscript or the next move by your chess opponent (or a predator). In such cases, not even the most complex stimulus-response programs (learned or innate) will help an animal in evading the undesired surprises and obtaining the desired ones. If the evasive actions taken by the female house fly were predictable, males could short cut and catch them with much less effort. It is essential to not leave the

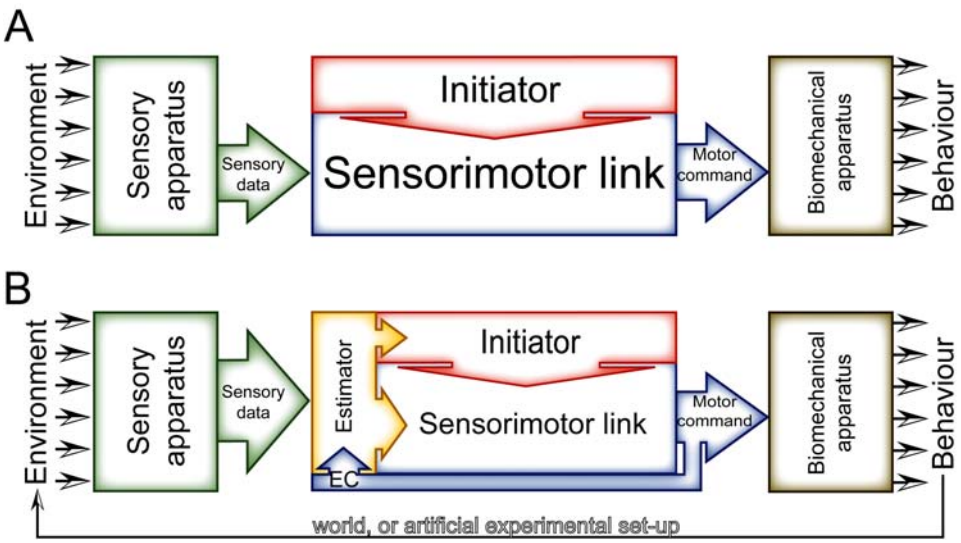


Figure 7. Suggested models for open-and closed-loop experiments. A–Open-loop model as proposed in Fig. 1b (for the *openloop* group). B–Closed-loop model (for the *onestripe* and *uniform* groups). Performance in a situation with a closed reafferent feedback loop is commonly modeled with a state estimator, cross-correlating sensory input with recent motor commands via an efference copy (EC). Such an evaluation is required for efficient behavioral control of incoming sensory data.
doi:10.1371/journal.pone.0000443.g007

generation of behavioral variability to chance (i.e., noise), but to keep it under neural control (i.e., nonlinearity). As such, evolution can fine-tune the balance between sensorimotor mapping and superimposed indeterminacy, defining the required compromise between spontaneous and reactive behavior. The variability of systems under tight constraints will be explained mostly by noise (because the variability under neural control is minimized, such as escape and pursuit responses in flies) [76], whereas noise may play a very small role in generating variability of less constrained behaviors (such as the ones observed here or the evasive actions taken by female house flies) [19,20,77]. This notion of brains operating on the critical edge between determinism and chaos has also been used to describe human magnetoencephalographic recordings [78]. Analogous to Heisenberg's uncertainty principle [79,80], much behavioral variability arises not out of practical constraints, but out of the principles of evolved brain function. In "*What is Life?*" Erwin Schrödinger claimed that fundamental indeterminism would never arise in the living world [81]. Today however, the picture emerges that as much as simple taxis, mate pursuit or course control require deterministic sensorimotor programs [7,13,14,56,57,76], more complex interactions require behavioral indeterminism, as evidenced by recent studies in game theory [61,63,65], exploration/foraging behavior [71], feeding [82] and pursuit-evasion contests ("Protean Strategy") [19,23,77,83]. Clearly, deterministic behavior will be exploited [23,84] and leaves us helpless in unpredictable situations [30,85]. Brains indeed do throw the dice–but by refuting the notion of stochasticity our results imply that they have exquisite control over when, where and how the dice are thrown [86].

Spontaneity is the basis for operant behavior

If unpredictability is so important, why is the 'random number generator' in the fly brain not perfect? For one, perfect unpredictability might not be required for survival. In addition, variable behavior might serve a second function. Variable, spontaneous behavior is the only way to find out which portions of the incoming sensory stream are under operant control by the animal's behavior. If much of the variation in this stream is due to random noise (i.e., Gaussian), behaving in a non-Gaussian way may aid in the detection of those variations which can be brought under behavioral control. Given these considerations and that our data imply a memory for past events influencing behavior initiation, it is tempting to perceive such mechanisms of spontaneous behavior initiation as the basis for operant behavior, operant conditioning and habit formation [74]. Following this notion, the ecologically so advantageous heavy-tailed searching strategy may be brought about by constantly engaging motor outputs and monitoring their effects in a decision-based queuing process. Such a process prioritizes certain items in a list over others (for instance yaw turns over thrust control, roll or proboscis extension) and has been shown to lead to heavy-tailed behavior patterns [33,67]. These considerations lend credence to an early, rarely cited cognitive hypothesis on the significance of behavioral variability in vertebrates [28] and suggest that it is actually much more profoundly valid throughout the taxa, with the prospect of studying its biological basis in a genetically tractable model system. Identifying the neural circuitry housing the initiator will be the logical next step in this research.

METHODS

Drosophila at the torque compensator

Flies Flies are kept on standard cornmeal/molasses medium [28] at 25°C and 60% humidity with a 14 hr light/10 hr dark regime. Females aged 24–48 h are briefly immobilized by cold-anaesthesia and glued (Loctite UV glass glue) with head and thorax to a triangle-shaped copper hook (diameter 0.05 mm) the day before the experiment. The animals are then kept individually overnight in small moist chambers containing a few grains of sucrose.

Experiments Fly yaw torque behavior was recorded using a torque compensator [87] with each fly flying stationarily in a vertical drum (arena) as described before [35,88] for 30 minutes. The *Drosophila* flight simulator is a computer controlled feedback system in which the fly uses its yaw torque to control the rotations of a panorama surrounding it (Fig. 2, Video S1). The core device is the torque meter [35,89–91], which measures a fly's angular momentum around its vertical body axis. The fly, glued to the hook, is attached to the torque meter via a clamp to accomplish stationary flight in the centre of a cylindrical panorama (arena; diameter 58 mm), homogeneously illuminated from behind (Fig. 2). The light source is a 100W, 12V tungsten-iodine bulb.

In the case that the feedback loop between the fly's behavior and its environment is open (i.e., "open loop"), the arena is empty, stationary and thus supplying a visually constant environment (white light). The fly is stationary, providing for a stable environment in terms of volatiles (odours) and magnetic or electrostatic fields. Any potential auditory stimuli are uncontrolled and bear no correlation to the fly's behavior. An analog to digital converter card (PCL812; Advantech Co.) feeds the yaw torque signal into a computer which stores the trace (sampling frequency 20Hz) for later analysis. 13 flies from this condition form the group "*openloop*".

In addition to the *openloop* group, we have analyzed data from two control groups. These groups controlled arena positioning with the operant feedback loop between behavior and arena closed. In "closed-loop", the situation is similar, but differs in that the arena carries either a single stripe ("*onestripe*") or is uniformly dashed ("*uniform*"). In these cases, a computer controlled electric motor rotates the arena such that its angular velocity is proportional to, but directed against the fly's yaw torque (coupling factor $K = -11^\circ/s \cdot 10^{-10}$Nm). This enables the fly to stabilize the panorama and to control its angular orientation. Each of the two groups contains the data from 13 flies. Only 30 minute-long uninterrupted flights in the respective situation are included in the analyses.

Data series

Yaw torque traces Observing the stored yaw torque traces after the experiment (Fig. S1), it becomes apparent that the behavioral output does not reflect the constancy of the environmental input at all. Instead, the yaw torque signal shows large fluctuations over the entire yaw torque range. In the *openloop* group, there are two sorts of fluctuations: baseline fluctuations and torque spikes. Because of the lack of landmarks, the fly is unable to acquire optomotor balance in order to fly straight, whereas in the two other groups, the pattern(s) on the arena enable straight flight and a constant baseline in optomotor balance (Fig. S1).

Torque spikes In free flight, fruit flies alter flight direction using rapid stereotyped turns termed body saccades [35,88]. Such saccades can alter flight direction by up to 90° in 50ms with turning velocities exceeding 1000°/s [14,31,35,92–96]. The flight path between saccades is comparatively straight [97]. At the torque compensator, these saccades manifest themselves as short bursts of torque ("spikes"). The dynamics of the spikes themselves adjust to tethered flight conditions, but otherwise tethered flight is in many ways very similar to free flight [14,35,96]. After low-pass

filtering the raw data (6^{th} order Butterworth IIR, passband 6 Hz, stopband 9 Hz) to remove measurement noise, the zero-crossings of the gradient are detected. The time of the zero-crossing is qualified as a spike event if the peak amplitude falls above a given threshold and outside of a given refractory period after the last spike. The time between two successive spikes is stored as inter-spike-interval (ISI). For each detected spike, the direction (left-turning or right-turning) is stored as well (see Fig. S2). A lower cut-off is made at 300 detected spikes to be able to perform meaningful mathematical analysis, discarding all animals failing this criterion. This, as well as all of the following algorithms was implemented in Matlab (The Mathworks Inc., Natick MA, USA).

Computer-generated control series All our algorithms were also applied to computer-generated random ISI series. Standard stochastics predict the outcome of each algorithm for this group of ISI series, which thus provides a valuable control group. For each of the 13 animals from the *openloop* group, a Poisson distribution was fitted to the ISI histogram. Random series with identical length to the *openloop* series were generated by drawing from these distributions, forming the "*poisson*" group.

Releasing the restriction of a constant spike rate, we generated data using a doubly stochastic Poisson process (or *Cox* process) [14,35,96]. For each fly from the *openloop* group, we estimated the instantaneous spike rate for each ISI_i by $1/(ISI_i\text{-}ISI_{i-1})$. The distribution of this top-level stochastic process was modeled non-parametrically, i.e. by computing histograms (bin size 10). To generate test data successive values were drawn at random from this top-level distribution. Each randomly drawn value provided the rate for the bottom-level Poisson process generating torque spikes. This process was iterated until the number of ISIs matched the corresponding fly sequence. Thus, both first and second-order statistics were matched in he *openloop* and the *cox* series.

As a model for a more complex composite stochastic process we used a branching Poisson process (BPP) [36,37]. There are many variants of such composite processes and a number of them are known to generate heavy-tailed probability distributions like the ones we observed in the fly groups. Specifically, we implemented a series cascade of Thomas processes (Fig. S4a): A top-level Poisson process with a constant rate generates a series of events. This series of singular events is filtered through a filter yielding a continuously valued, time varying signal. This is used as the rate for a (non-homogeneous) Poisson process on the next level, which also generates a series of events. This scheme is iterated over all levels. The output of all levels is combined to yield the output of the BPP (hence *branching* PP). For our analyses we generated data using a BPP comprising 10 levels and an initial rate of 0.05. The transfer function of the filter is given by the coefficients [1] in the nominator and [1–0.9] in the denominator, yielding an exponentially decaying impulse response function. Alternatively we used a 5-tap boxcar filter to investigate the effect of (non-)linearity on the properties of the data generated by the BPP.

In addition to ISI time series, we also computer-generated four categories of raw data traces for the nonlinear forecasting procedures:

I. A noisy sine function was used for comparison to a linear process. Data of the same length n as the yaw torque data were generated by

$$y_i = \sin\left(\frac{i}{2\pi}\right) + \sigma\xi_i + 2, \quad 0 \le i < n \tag{1}$$

with noise ξ_i drawn from a uniform distribution in the interval $[-1, 1]$. We set σ to 0.2.

II. For comparison to a process with known nonlinear properties we used the logistic map:

$$y_i = (\mu + \sigma\xi_i) y_{i-1}(1 - y_{i-1}), \quad 1 \le i \le n. \tag{2}$$

We chose $\mu = 3.9$, $\sigma = 0.1$, and initialized y_0 to a random value in the interval [0, 1].

III. We adapted a model designed to simulate spontaneous search behavior as an example for modern autonomous, nonlinear agents. The original model [48] consisted of three coupled nonlinear oscillators and a sensory organ. Two oscillators provided output for left and right turns, respectively. The remaining oscillator provided activating input for the other two oscillators. To model open loop behavior where sensory input is constant, we removed the sensory input from the model (*automat*; Fig. S4b).

The state s_i^o of oscillator o ($o \in \{R, L, A\}$ for left, right, and activating) at time point i is given by

$$s_i^o = \lambda_i^o s_{i-1}^o \left(1 - s_{i-1}^o\right), \quad 1 \le i \le n. \tag{3}$$

The initial state $s^o{}_0$ of an oscillator is randomly chosen in the interval [0,1]. We re-set s_i^o to 10^{-6} whenever it falls below this value.

The parameters λ^o evolve according to

$$\begin{aligned} \lambda_i^A &= \mu + \sigma\eta_i^A \\ \lambda_i^L &= \mu + \sigma\eta_i^L + s_i^A - \alpha s_i^R \\ \lambda_i^R &= \mu + \sigma\eta_i^R + s_i^A - \alpha s_i^L. \end{aligned} \tag{4}$$

Here, η^o is Gaussian noise in the interval $[-1, 1]$. The model parameter μ controls the behavior of the logistic maps. The term $\sigma\eta_i^o$ acts as a perturbation on μ. The parameter α controls the strength of the inhibition between the left and right turn oscillators. The simulated torque signal y is computed by

$$y_i = s_i^L - s_i^R. \tag{5}$$

The model parameters μ, σ, and α were adjusted in the following ways to generate a number of different *automat* simulations. At first, the parameters were chosen according to the original publication ($\mu = 1.1$, $\sigma = 1.1$, and $\alpha = 1$; *automat* in Fig. S5). From there, parameters were explored and adjusted manually until the output appeared to be indistinguishable from fly yaw torque data ($\mu = 1.1$, $\sigma = 0.75$, and $\alpha = 1.15$; *automat 2* in Fig. 6). For this simulation, the previous time-step was also added to the current state (i.e., $s^A{}_{i-1} + s_i^A$), simulating a one-step memory. Next, μ was increased and σ decreased to bring the agent beyond the point of stability ($\mu = 3.4$, $\sigma = 0.3$, and $\alpha = 3.4$, *automat 1* in Fig. 6).

Mathematical analyses

In a stepwise fashion we tested increasingly more sophisticated models, eliminating the less complex models at each step.

Geometric Random Inner Products (GRIP) The GRIP formalism has been developed to quantify the performance of random number generators [55]. It is based on the observation that the average inner product of randomly distributed vectors in n-dimensional geometric objects (like hyper-spheres or hyper-cubes) converges to object specific constants. The deviation from this constant can be used as a measure for the randomness of

a sequence. One application was studying the randomness of the digits of π [34].

Here we apply GRIP to quantify the randomness of ISI sequences. In a first step, the ISI sequence (l_1, l_2, … l_n) is embedded in an d-dimensional space such that

$$\mathbf{v}_1 = \begin{pmatrix} l_1 \\ l_2 \\ \vdots \\ l_d \end{pmatrix}, \mathbf{v}_2 = \begin{pmatrix} l_{d+1} \\ l_{d+2} \\ \vdots \\ l_{2d} \end{pmatrix}, \ldots \mathbf{v}_m = \begin{pmatrix} l_{(m-1)d+1} \\ l_{(m-1)d+2} \\ \vdots \\ l_{(m-1)d+d} \end{pmatrix} m = \lfloor \frac{n}{d} \rfloor d \quad (6)$$

are vectors which are presumed to be random. For three consecutive vectors $\mathbf{v}_i$, $\mathbf{v}_{i+1}$, $\mathbf{v}_{i+2}$ the differences $\mathbf{v}_{12} = \mathbf{v}_{i+1} - \mathbf{v}_i$ and $\mathbf{v}_{23} = \mathbf{v}_{i+2} - \mathbf{v}_{i+1}$ are computed. The average inner product of these vectors has been shown to converge to a geometric constant c_d, i.e.

$$\langle \mathbf{v}_{12} \cdot \mathbf{v}_{23} \rangle_d = c_d. \quad (7)$$

For an exponential probability density function $p(l) = e^{-al}$ of ISIs of length l, this constant is

$$c_d = -\frac{d}{a^2} \quad (8)$$

(Tu, S.J.; personal communication). We set the embedding dimension $d = 3$. Exponential functions were fitted to the ISI histograms, and the geometric constants c_d were determined for each fly. To compare the randomness between groups, we computed the absolute differences between the left and right side of eq. (7) in terms of standard deviations of the left side. The results were averaged for each group.

Exponential distributions We compared ISI series to exponential distributions by first fitting an exponential distribution to the ISI series and then plotting the ISI series on a semi-logarithmic scale with the fitted exponential as a straight line. Wherever the ISI series deviates from the straight line, it deviates from an exponential distribution with the same rate.

Lévy exponent If the distribution of ISIs of duration l can be characterized by a probability density function

$$p(l) \sim l^{-\mu} \quad (9)$$

with $1 < \mu \leq 3$, the distribution is called a Lévy distribution. In contrast to Gaussian or Poisson distributions of step lengths, in Lévy motion small steps are more often interspersed with longer steps, causing the variance of the distribution to diverge. Additionally, Lévy distributions are self-similar at all scales or, in other words, the step lengths have no characteristic scale [98]. Lévy distributions are commonly found in animal behavioral patterns [99]. For foraging behavior it can be shown that $\mu \approx 2$ results in an optimal coverage of an area with randomly located target sites if the global site concentration is low [39]. We determined Lévy exponents by fitting straight lines to log-log plots of ISI histograms:

$$\mu = -\frac{d \log N(l)}{d \log l} \quad (10)$$

Here, $N(l)$ is the number of ISIs in the bin representing duration l. All single fly series within one group were concatenated and μ computed as a single value for each group.

Correlation dimension To evaluate the possibility that the apparently random ISI sequences are produced by a nonlinear system causing chaotic dynamics we estimated the fractal dimensions of the underlying attractor of the sequences. Specifically, we computed the limit of the correlation dimension v for an increasing dimensionality d of the embedding space [100],

$$\lim_{d \to \infty} v_d = D, \quad (11)$$

where D is the fractal dimension of the chaotic attractor. The correlation dimension is given by:

$$C_d(\varepsilon) = \varepsilon^{v_d} \quad (12)$$

C_d is the correlation integral and measures how frequently the system state returns into a vicinity of size ε,

$$C_d(\varepsilon) = \frac{\left| \left\{ (\mathbf{v}_i, \mathbf{v}_j), |\mathbf{v}_i - \mathbf{v}_j| \leq \varepsilon \right\} \right|}{m(m-1)}, \quad (13)$$

where vectors $\mathbf{v}_{1\ldots m}$ are the embedded ISI sequence of dimensionality d. Similarly, we computed the limit of the information dimension $\lim_{d \to \infty} \delta_d$ defined as[101]:

$$H_d(\varepsilon) = \varepsilon^{\delta_d}. \quad (14)$$

H_d is the entropy of the system in phase space and can be written as

$$H_d(\varepsilon) = -\sum_{i=1}^{N(\varepsilon)} p_i \log p_i. \quad (15)$$

Here, p_i is the probability that the system is in state i represented by cubes of size ε in the state space. Numerically, correlation and information dimension were determined by fitting lines into log-log plots of the correlation integral and the entropy, respectively.

For random sequences the correlation dimension diverges. Since we observe convergence for our ISI sequences, we use this as another indicator that they are not trivially random. In order to exclude more complex stochastic processes, we compared the correlation dimension for each dataset with the values obtained from surrogate data. Surrogate datasets were created by randomly shuffling the ISIs of the measured sequence. This retains the first order statistics but destroys any dynamic information depending on the history of the system. If the correlation dimension of the measured sequence and the surrogate data differ significantly, we can conclude that the sequence contains dynamic information. To evaluate the difference we computed a normalized histogram of correlation dimensions of $N = 1000$ surrogate datasets. In this histogram, the value at the position of the correlation dimension of the measured sequence corresponds to the probability to obtain this value by a random sequence with the same first order statistics. These probabilities were averaged across individuals for each group.

Root-mean-square fluctuation of displacement To detect long-range correlations in our ISI series, we applied a method based on the root mean square (r.m.s.) fluctuation of displacement [102]. If (l_1, l_2, … l_n) is a sequence of ISIs, the net displacement $y(t)$ is defined as the running sum $y(t) = \sum_{i=1}^{t} l_i$. The fluctuation of

displacement is defined as $\Delta y(t) \equiv y(t_0+t) - y(t_0)$, and the statistical measure characterizing the series is the root of the mean squares

$$F(t) = \sqrt{\langle (\Delta y(t))^2 \rangle - \langle \Delta y(t) \rangle^2}. \quad (16)$$

The angular brackets denote expectation value over all possible values t_0. The r.m.s. fluctuation obeys a power law, i.e.

$$F(t) \propto t^{\alpha}. \quad (17)$$

Uncorrelated time series yield $\alpha = 1/2$, as do Markov processes for sufficiently large t. Processes with long-range correlations yield $\alpha \neq 1/2$. We plotted $F(t)$ for each ISI series on a double logarithmic scale, fitted straight lines and calculated the regression slope α to obtain one value for each series which was then averaged for each group.

Simplex projection Simplex projection [49] is a nonlinear method for making short-term forecasts of time series. The quality of the forecast is measured by computing the correlation coefficient between the forecast and the original series. Depending on the nature of the data, the evolution of the correlation coefficient shows different developments for increasing forecasting intervals. For a linear, but noisy process the correlation coefficient decreases only slowly with increasing prediction intervals. In contrast, a chaotic process is characterized by a fast decay of prediction accuracy. One of the great advantages of this method is that it can be applied to short series, such as our data. Raw yaw torque data were detrended by taking the first difference of the series. ISI series were not detrended.

The method starts by embedding the ISI or data sequence in a d-dimensional space. Unlike the embedding used for GRIP and correlation dimension, where each ISI is used in only one vector, here each ISI appears in d vectors. Specifically, the embedding is now

$$\mathbf{v_1} = \begin{pmatrix} l_1 \\ l_2 \\ \vdots \\ l_d \end{pmatrix}, \mathbf{v_2} = \begin{pmatrix} l_2 \\ l_3 \\ \vdots \\ l_{d+1} \end{pmatrix}, \ldots \mathbf{v_m} = \begin{pmatrix} l_{n-d+1} \\ l_{n-d+2} \\ \vdots \\ l_n \end{pmatrix} m = n-d+1. \quad (18)$$

The resulting set of points in d-dimensional space is split in two halves, the library set $\mathbf{L}$ and the prediction set $\mathbf{P}$. We consider each vector $\mathbf{p}^t \in \mathbf{P}$ as composed of a consecutive sequence of d observed ISI points. From this sequence a prediction about the following ISI durations ($T_p = 1, 2, \ldots$) is to be generated. From eq. (18) can be seen, that if $\mathbf{p}^t = \mathbf{p}_i$ is the i-th vector in the prediction set, the observed ISI T_p steps ahead is $\boldsymbol{p}^{t+T_p}(d) = \boldsymbol{p}_{i+T_p}(d)$, e.g. the prediction for the sequence in $\mathbf{v}_1$ one step ahead is $\mathbf{v}_2(d)$.

To generate a prediction for a vector $\mathbf{p}^t$ from the prediction set, its d+1 nearest neighbors $\mathbf{l}^t_1 \ldots \mathbf{l}^t_{d+1} \in \mathbf{L}$ are selected. Associated with each neighbor is a weight

$$w_i = \frac{1}{\sum_{j=1}^{d+1} \exp\left(- \frac{\|\boldsymbol{p}^t - \boldsymbol{l}^t_j\|}{\bar{w}} \right)} \exp\left(- \frac{\|\boldsymbol{p}^t - \boldsymbol{l}^t_i\|}{\bar{w}} \right),$$

$$\bar{w} = \frac{1}{d+1} \sum_{j=1}^{d+1} \left\| \boldsymbol{p}^t - \boldsymbol{l}^t_j \right\|.$$

A prediction $\hat{\boldsymbol{p}}^{t+T_p}$ for $\mathbf{p}^t$ after T_p steps is then given by the weighted superposition of the evolution of the neighbors after T_p time steps, i.e.

$$\hat{\boldsymbol{p}}^{t+T_p} = \sum_{j=1}^{d+1} w_j \boldsymbol{l}_j^{t+T_p}. \quad (20)$$

Returning back from the embedding space to the temporal domain of the sequence, we consider the predicted ISI,

$$\hat{p}^{t+T_p} = \hat{\boldsymbol{p}}^{t+T_p}(d), \quad (21)$$

i.e. the last component of vector $\hat{\boldsymbol{p}}^{t+T_p}$, and compare it with the observed ISI T_p steps ahead, which is given by

$$p^{t+T_p} = \boldsymbol{p}^{t+T_p}(d). \quad (22)$$

The coefficient of correlation between the sequence of predicted ISIs and the true values is then used as a measure for the prediction accuracy.

S-map procedure The S-map procedure (sequentially locally weighted global linear map [52]) is in many respects similar to the simplex projection. Here, instead of looking at the evolution of only the nearest neighbors to generate a prediction, *all* vectors in the library set are used. A single linearity parameter θ controls if the influence of the library vectors is linear ($\theta = 0$) or nonlinearly weighted by their respective distance to the vector used for the prediction. To apply the denotation used for the simplex projection, the prediction $\hat{p}^{t+Tp}$ from a vector $\mathbf{p}^t \in \mathbf{P}$ is now given by

$$\hat{p}^{t+T_p} = \boldsymbol{c} \cdot \boldsymbol{p}^t, \quad (23)$$

where $\mathbf{c}$ is a weight vector that is newly computed for every prediction $\mathbf{p}^t$. It is the solution of

$$\boldsymbol{b} = \boldsymbol{A}\boldsymbol{c}, \quad (24)$$

where the rows of matrix $\mathbf{A}$ contain the library vectors $\mathbf{l}$ and vector $\mathbf{b}$ the corresponding, observed ISI duration T_p time steps after the sequence contained in $\mathbf{l}$. Formally, $\mathbf{A}$ and $\mathbf{b}$ are given by

$$\boldsymbol{b}(i) = w(\boldsymbol{l}_i, \boldsymbol{p}^t) \boldsymbol{l}_{i+Tp}(d), \boldsymbol{A}_{ij} = w(\boldsymbol{l}_i, \boldsymbol{p}^t) \boldsymbol{l}_i(j) 1 \leq i \leq |\boldsymbol{L}|, 1 \leq j \leq d. \quad (25)$$

As can be seen, the number of rows in $\mathbf{A}$ (and the length of $\mathbf{b}$) is equal to the size of the library set $|\mathbf{L}|$, which in most cases will be larger than the embedding dimension d. Therefore, eq. (24) will be over-determined and singular value decomposition (SVD) is used to obtain an optimal solution.

Function w is used to weight the library vectors by their distance to the prediction vector:

$$w(\mathbf{v}_1, \mathbf{v}_2) = \exp\left(-\theta \frac{\|\mathbf{v}_1 - \mathbf{v}_2\|}{\bar{w}} \right), \bar{w} = \frac{1}{|\boldsymbol{L}|} \sum_{i=1}^{|\boldsymbol{L}|} \|\boldsymbol{l}_i - \boldsymbol{p}^t\|. \quad (26)$$

For $\theta = 0$, a linear map is obtained. Increasing θ puts more and more emphasis on library vectors close to the prediction vector.

As for the simplex projection, the accuracy of predictions is evaluated by the correlation coefficient between the predicted and the observed series.

Statistical evaluation

To test for significant differences between several groups, we first used a Kruskal-Wallis ANOVA to test the hypothesis that all groups were drawn from the same population. If this hypothesis was rejected, 2-tailed post-hoc tests provided information as to the source of the differences. These tests were conducted for GRIP values (Fig. 3) and the probabilities to obtain the original correlation dimension with shuffled data (Fig. 4). T-tests against single values were used to test individual groups against an expected value and Mann-Whitney U-Tests for pairwise comparisons (Fig. 5).

SUPPORTING INFORMATION

Figure S1 Example yaw torque traces. Left column-total traces. Right column-magnified section from minutes 5-10 of the total traces. Red lines delineate enlarged sections. Upper row is from an animal flying in open loop in a featureless, white panorama (openloop). The middle row is from an animal flying in closed loop in a panorama with a single black stripe (onestripe). The lower row is from an animal flying in closed loop in a uniformly dashed arena (uniform).
Found at: doi:10.1371/journal.pone.0000443.s001 (0.68 MB TIF)

Figure S2 Descriptive statistics of spiking behavior. A-The probability to perform consecutive spikes in the same direction. Random spike directions show equal probability for left and right turns, while fly data are dependent on the environmental situation of the fly. Flies fixate a single stripe and hence produce alternating spikes to keep the stripe in front of them. The onestripe group therefore is more similar to the poisson group than the other fly groups. Flies in uniform environments show persistent turning direction over several consecutive spikes. These spike trains in the same direction can be interpreted as search spirals. B-Total number of spikes. Openloop and poisson show the same values, because poisson was generated by drawing series with the same length as those in openloop. The onsestripe group shows fewer spikes, because of the long intervals flying straight towards the stripe.
Found at: doi:10.1371/journal.pone.0000443.s002 (0.89 MB TIF)

Figure S3 Log-linear plots of fly and Poisson data. Corroborating the results from our GRIP analysis, exponential distributions (straight black lines) cannot be fitted to fly ISI series, whereas the poisson series shows the expected exponential distribution. Fly ISI series all show an excess of long intervals, suggesting a heavy-tailed distribution. See Methods for details.
Found at: doi:10.1371/journal.pone.0000443.s003 (0.38 MB TIF)

Figure S4 Schematic diagrams of complex stochastic and simple nonlinear models. A-The branching Poisson process (BPP) as an example for complex stochastic models. The BPP consists of cascading units of filter functions and Poisson processes. Each unit's filter function receives the events from the Poisson process upstream and drives the rate of the Poisson process associated with it. The (unfiltered) output of all Poisson processes is combined to yield the total output of the model. B-The nonlinear automat is an example how simple nonlinear processes can generate complex behavior. The activator sends excitatory input to both turn generators. The turn oscillators inhibit each other. The output is the difference signal between the left and right turn oscillator. Each oscillator is described by a logistic map, and the coupling modulates the individual parameters of each map. See Methods for details.
Found at: doi:10.1371/journal.pone.0000443.s004 (1.44 MB TIF)

Figure S5 S-Map analysis of all fly data and additional control series. A-S-Map analysis of ISI series. Depicted are the averaged results for the three fly groups. Interestingly, the fly group with a singularity in the environment (onestripe) can be clearly distinguished from the two groups with uniform environment (openloop and uniform). Note that the closed-loop groups (onestripe and uniform) also exhibit the nonlinear signature, excluding the possibility that the variability is an artefact of the constant stimulus situation in the openloop group. B-S-Map analysis of raw data series. At high parameter values, the logistic map shows the typical increase in forecast skill with increasingly nonlinear models, while the noisy sine function does not show any such improvement. The nonlinear agent (automat) with the originally published parameters behaves almost randomly, despite the nonlinear mechanisms generating the output. The fly data come to lie in-between the extreme control data, showing both an increase in forecast skill with increasingly nonlinear models and moderate overall correlation coefficients.
Found at: doi:10.1371/journal.pone.0000443.s005 (0.42 MB TIF)

Video S1 Tethered Drosophila. Tethered flying Drosophila can beat its wings, move its abdomen, legs and proboscis, but cannot rotate or otherwise move.
Found at: doi:10.1371/journal.pone.0000443.s006 (1.94 MB AVI)

ACKNOWLEDGMENTS

We are grateful to Martin Heisenberg, Randolf Menzel, Juliane Rama, Tilman Franke, Carsten Duch, Peter Wolbert, Anton Bovier and Randy Gallistel for critically commenting on an earlier version of the manuscript. We are especially indebted to Shu-Ju Tu and Ephraim Fischbach for assistance implementing the GRIP method and to Mark Frye for reminding BB of the main issue and thereby jump-starting this work. Gonzalo Garcia de Polavieja improved the manuscript tremendously by introducing us to the complex stochastic processes described by Cox, Teich and others.

Author Contributions

Conceived and designed the experiments: BB. Performed the experiments: BB. Analyzed the data: BB AM. Contributed reagents/materials/analysis tools: BB GS AM CH. Wrote the paper: BB. Other: Conceived the study, selected the analytical tools, conducted the behavioral experiments: BB. Contributed crucially to the implementation of the S-Map procedure, its interpretation and cross-checked selected results in blind; read and commented on the paper: CH GS. Performed all mathematical analyses and advised B.B. on their selection, read and commented on the paper: AM CH.

REFERENCES

1. Laplace PS (1825) Essai Philosophique sur les Probabilités. Paris: Gauthier-Villars.
2. Malescio G (2005) Predicting with unpredictability. Nature 434: 1073.
3. Garland B (2004) Neuroscience and the Law: Brain, Mind, and the Scales of Justice. Chicago: University of Chicago Press.
4. Pavlov IP (1927) Conditioned reflexes. Oxford: Oxford University Press.
5. Skinner BF (1974) About Behaviorism. New York, NY, USA: Knopf.
6. Dickinson A (1985) Actions and Habits-the Development of Behavioral Autonomy. Philos Trans R Soc Lond B Biol Sci 308: 67–78.
7. Webb B (2002) Robots in invertebrate neuroscience. Nature 417: 359–363.

8. Abbott A (2007) Biological robotics: Working out the bugs. Nature 445: 250–253.
9. Franceschini N, Ruffier F, Serres J (2007) A bio-inspired flying robot sheds light on insect piloting abilities. Curr Biol 17: 329–335.
10. Mobbs D, Lau HC, Jones OD, Frith CD (2007) Law, Responsibility, and the Brain. PLoS Biology 5: e103.
11. Greene J, Cohen J (2004) For the law, neuroscience changes nothing and everything. Philos Trans R Soc Lond B Biol Sci 359: 1775–1785.
12. Mauk MD (2000) The potential effectiveness of simulations versus phenomenological models. Nat Neurosci 3: 649–651.
13. Bülthoff H, Götz KG (1979) Analogous motion illusion in man and fly. Nature. pp 636–638.
14. Frye MA, Dickinson MH (2004) Closing the loop between neurobiology and flight behavior in Drosophila. Curr Opin Neurobiol 14: 729–736.
15. Lowen SB, Ozaki T, Kaplan E, Saleh BEA, Teich MC (2001) Fractal features of dark, maintained, and driven neural discharges in the cat visual system. Methods 24: 377–394.
16. Stein RB, Gossen ER, Jones KE (2005) Neuronal variability: noise or part of the signal? Nat Rev Neurosci 6: 389–397.
17. Briggman KL, Abarbanel HD, Kristan WB Jr (2005) Optical imaging of neuronal populations during decision-making. Science 307: 896–901.
18. Brembs B, Lorenzetti FD, Reyes FD, Baxter DA, Byrne JH (2002) Operant reward learning in Aplysia: neuronal correlates and mechanisms. Science 296: 1706–1709.
19. Grobstein P (1994) Variability in behavior and the nervous system. In: Ramachandran VS, ed (1994) Encyclopedia of Human Behavior. New York: Academic Press. pp 447–458.
20. Glimcher PW (2005) Indeterminacy in brain and behavior. Annu Rev Psychol 56: 25–56.
21. Raichle ME (2006) NEUROSCIENCE: The Brain's Dark Energy. Science 314: 1249–1250.
22. Crabbe JC, Wahlsten D, Dudek BC (1999) Genetics of mouse behavior: interactions with laboratory environment. Science 284: 1670–1672.
23. Miller GF (1997) Protean primates: The evolution of adaptive unpredictability in competition and courtship. In: Whiten A, Byrne RW, eds (1997) Machiavellian Intelligence II: Extensions and evaluations. Cambridge: Cambridge University Press. pp 312–340.
24. de Ruyter van Steveninck RR, Lewen GD, Strong SP, Koberle R, Bialek W (1997) Reproducibility and Variability in Neural Spike Trains. Science 275: 1805–1808.
25. Ma WJ, Beck JM, Latham PE, Pouget A (2006) Bayesian inference with probabilistic population codes. Nat Neurosci 9: 1432–1438.
26. Smolen P, Baxter DA, Byrne JH (2000) Mathematical modeling of gene networks. Neuron 26: 567–580.
27. Marder E, Goaillard J-M (2006) Variability, compensation and homeostasis in neuron and network function. Nature Reviews Neuroscience 7: 563–574.
28. Krechevsky I (1937) Brain mechanisms and variability II. Variability where no learning is involved. J Comp Physiol Psychol 23: 139–160.
29. Ashwin P, Timme M (2005) Nonlinear dynamics: when instability makes sense. Nature 436: 36–37.
30. Heisenberg M (1994) Voluntariness (Willkürfähigkeit) and the general organization of behavior. L Sci Res Rep 55: 147–156.
31. Fry SN, Sayaman R, Dickinson MH (2003) The Aerodynamics of Free-Flight Maneuvers in Drosophila. Science 300: 495–498.
32. Reynolds A, Frye M (2007) Free-Flight Odor Tracking in Drosophila Is Consistent with an Optimal Intermittent Scale-Free Search. PLoS ONE 2: e354.
33. Barabasi A-L (2005) The origin of bursts and heavy tails in human dynamics. Nature 435: 207–211.
34. Tu SJ, Fischbach E (2003) Geometric random inner products: A family of tests for random number generators. Physical Review E 67.
35. Heisenberg M, Wolf R (1984) Vision in *Drosophila*. Genetics of Microbehavior. Berlin, Heidelberg, New York, Tokio: Springer. pp 1–250.
36. Cox DR (1955) Some Statistical Methods Connected with Series of Events. Journal of the Royal Statistical Society Series B-Statistical Methodology 17: 129–164.
37. Cox DR, Isham V (1980) Point processes. Monographs on applied probability and statistics. London: Chapman and Hall.
38. Lowen SB, Teich MC (1991) Doubly Stochastic Poisson Point Process Driven by Fractal Shot Noise. Physical Review A 43: 4192–4215.
39. Viswanathan GM, Buldyrev SV, Havlin S, da Luz MG, Raposo EP, et al. (1999) Optimizing the success of random searches. Nature 401: 911–914.
40. Cole BJ (1995) Fractal Time in Animal Behavior-the Movement Activity of Drosophila. Anim Behav 50: 1317–1324.
41. Martin JR, Faure P, Ernst R (2001) The power law distribution for walking-time intervals correlates with the ellipsoid-body in Drosophila. J Neurogenet 15: 205–219.
42. Viswanathan GM, Afanasyev V, Buldyrev SV, Havlin S, da Luz MGE, et al. (2001) Levy fights search patterns of biological organisms. Physica A 295: 85–88.
43. Mantegna RN, Stanley HE (1995) Scaling behaviour in the dynamics of an economic index. Nature 376: 46–49.
44. Segev R, Benveniste M, Hulata E, Cohen N, Palevski A, et al. (2002) Long Term Behavior of Lithographically Prepared In Vitro Neuronal Networks. Physical Review Letters 88: 118102.
45. Fox Keller E (2007) A clash of two cultures. Nature 445: 603–603.
46. Grassberger P, Procaccia I (1983) Measuring the strangeness of strange attractors. Physica D: Nonlinear Phenomena 9: 189–208.
47. Darbin O, Soares J, Wichmann T (2006) Nonlinear analysis of discharge patterns in monkey basal ganglia. Brain Res 1118: 84–93.
48. Teich MC, Saleh BEA (2000) Branching processes in quantum electronics. IEEE J Sel Top Quantum Electron 6: 1450–1457.
49. Viswanathan GM, Afanasyev V, Buldyrev SV, Murphy EJ, Prince PA, et al. (1996) Levy flight search patterns of wandering albatrosses. Nature 381: 413–415.
50. Shannon CE, Weaver W (1963) Mathematical Theory of Communication: University of Illinois Press.
51. Wales DJ (1991) Calculating the Rate of Loss of Information from Chaotic Time-Series by Forecasting. Nature 350: 485–488.
52. Sugihara G, May RM (1990) Nonlinear Forecasting as a Way of Distinguishing Chaos from Measurement Error in Time-Series. Nature 344: 734–741.
53. Hsieh CH, Glaser SM, Lucas AJ, Sugihara G (2005) Distinguishing random environmental fluctuations from ecological catastrophes for the North Pacific Ocean. Nature 435: 336–340.
54. Dixon PA, Milicich MJ, Sugihara G (1999) Episodic fluctuations in larval supply. Science 283: 1528–1530.
55. Nepomnyashchikh VA, Podgornyj KA (2003) Emergence of adaptive searching rules from the dynamics of a simple nonlinear system. Adapt Behav 11: 245–265.
56. Land MF, Collett TS (1974) Chasing Behavior of Houseflies (Fannia-Canicularis)-Description and Analysis. Journal of Comparative Physiology 89: 331–357.
57. Boeddeker N, Egelhaaf M (2005) A single control system for smooth and saccade-like pursuit in blowflies. J Exp Biol 208: 1563–1572.
58. Boeddeker N, Egelhaaf M (2003) Steering a virtual blowfly: simulation of visual pursuit. Proceedings of the Royal Society of London Series B-Biological Sciences 270: 1971–1978.
59. Sugihara G, Casdagli M, Habjan E, Hess D, Dixon P, et al. (1999) Residual delay maps unveil global patterns of atmospheric nonlinearity and produce improved local forecasts. Proc Natl Acad Sci USA 96: 14210–14215.
60. Sugihara G, Grenfell B, May RM (1990) Distinguishing error from chaos in ecological time series. Philos Trans R Soc Lond B Biol Sci 330: 235–251.
61. Platt ML (2004) Unpredictable primates and prefrontal cortex. Nat Neurosci 7: 319–320.
62. Sanfey AG, Rilling JK, Aronson JA, Nystrom LE, Cohen JD (2003) The neural basis of economic decision-making in the Ultimatum Game. Science 300: 1755–1758.
63. McNamara JM, Barta Z, Houston AI (2004) Variation in behaviour promotes cooperation in the Prisoner's Dilemma game. Nature 428: 745–748.
64. Glimcher PW, Rustichini A (2004) Neuroeconomics: the consilience of brain and decision. Science 306: 447–452.
65. Brembs B (1996) Chaos, cheating and cooperation: Potential solutions to the Prisoner's Dilemma. Oikos 76: 14–24.
66. Leopold DA, Logothetis NK (1999) Multistable phenomena: Changing views in perception. Trends in Cognitive Sciences 3: 254–264.
67. Oliveira JG, Barabasi AL (2005) Human dynamics: Darwin and Einstein correspondence patterns. Nature 437: 1251.
68. Brockmann D, Hufnagel L, Geisel T (2006) The scaling laws of human travel. Nature 439: 462–465.
69. Mason MF, Norton MI, Van Horn JD, Wegner DM, Grafton ST, et al. (2007) Wandering Minds: The Default Network and Stimulus-Independent Thought. Science 315: 393–395.
70. Bartumeus F, Peters F, Pueyo S, Marrase C, Catalan J (2003) Helical Levy walks: Adjusting searching statistics to resource availability in microzooplankton. Proc Natl Acad Sci USA 100: 12771–12775.
71. Belanger JH, Willis MA (1996) Adaptive control of odor-guided locomotion: Behavioral flexibility as an antidote to environmental unpredictability. Adapt Behav 4: 217–253.
72. Krichmar JL, Nitz DA, Gally JA, Edelman GM (2005) Characterizing functional hippocampal pathways in a brain-based device as it solves a spatial memory task. Proc Natl Acad Sci USA 102: 2111–2116.
73. Todorov E (2004) Optimality principles in sensorimotor control. Nat Neurosci 7: 907–915.
74. Heisenberg M, Wolf R, Brembs B (2001) Flexibility in a single behavioral variable of Drosophila. Learn Mem 8: 1–10.
75. Bongard J, Zykov V, Lipson H (2006) Resilient Machines Through Continuous Self-Modeling. Science 314: 1118–1121.
76. Osborne LC, Lisberger SG, Bialek W (2005) A sensory source for motor variation. Nature 437: 412–416.
77. Korn H, Faber DS (2005) The Mauthner cell half a century later: A neurobiological model for decision-making? Neuron 47: 13–28.
78. Bassett DS, Meyer-Lindenberg A, Achard S, Duke T, Bullmore E (2006) From the Cover: Adaptive reconfiguration of fractal small-world human brain functional networks. Proc Natl Acad Sci USA 103: 19518–19523.
79. Heisenberg W (1930) Physical Principles of Quantum Theory. New York: Dover.

80. Heisenberg W (1952) Philosophic Problems of Quantum Physics. Woodbridge, CT: Ox Bow.
81. Schrödinger E (1944) What is life? London: Cambridge University Press.
82. Lum CS, Zhurov Y, Cropper EC, Weiss KR, Brezina V (2005) Variability of swallowing performance in intact, freely feeding *Aplysia*. J Neurophysiol 94: 2427–2446.
83. Shultz S, Dunbar R (2006) Chimpanzee and felid diet composition is influenced by prey brain size. Biology Letters 2: 505–508.
84. Jablonski PG, Strausfeld NJ (2001) Exploitation of an ancient escape circuit by an avian predator: relationships between taxon-specific prey escape circuits and the sensitivity to visual cues from the predator. Brain Behav Evol 58: 218–240.
85. Greenspan RJ (2005) No Critter Left Behind: An Invertebrate Renaissance. Curr Biol 15: R671–R672.
86. Barinaga M (1996) Neuroscience: Neurons Put the Uncertainty Into Reaction Times. Science 274: 344–340.
87. Guo A, Liu L, Xia S-Z, Feng C-H, Wolf R, et al. (1996) Conditioned visual flight orientation in *Drosophila*; Dependence on age, practice and diet. Learning and Memory 3: 49–59.
88. Götz KG (1964) Optomotorische Untersuchung des visuellen Systems einiger Augenmutanten der Fruchtfliege Drosophila. Kybernetik 2: 77–92.
89. Wolf R, Heisenberg M (1991) Basic organization of operant behavior as revealed in Drosophila flight orientation. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 169: 699–705.
90. Brembs B, Hempel de Ibarra N (2006) Different parameters support generalization and discrimination learning in *Drosophila* at the flight simulator. Learn Mem 13: 629–637.
91. Brembs B, Wiener J (2006) Context generalization and occasion setting in *Drosophila* visual learning. Learn Mem 13: 618–628.
92. Heisenberg M, Wolf R (1979) On the fine structure of yaw torque in visual flight orientation of drosophila-melanogaster. J Comp Physiol A Sens Neural Behav Physiol 130: 113–130.
93. Heisenberg M, Wolf R (1988) Reafferent control of optomotor yaw torque in *Drosophila melanogaster*. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 163: 373–388.
94. Heisenberg M, Wolf R (1993) The sensory-motor link in motion-dependent flight control of flies. Rev Oculomot Res 5: 265–283.
95. Wolf R, Heisenberg M (1990) Visual control of straight flight in drosophila-melanogaster. J Comp Physiol A Sens Neural Behav Physiol 167: 269–284.
96. Mayer M, Vogtmann K, Bausenwein B, Wolf R, Heisenberg M (1988) Flight control during free yaw turns in Drosophila melanogaster. J Comp Physiol A Sens Neural Behav Physiol 163: 389–400.
97. Tammero LF, Dickinson MH (2002) The influence of visual landscape on the free flight behavior of the fruit fly Drosophila melanogaster. J Exp Biol 205: 327–343.
98. Tu SJ, Fischbach E (2005) A study on the randomness of the digits of pi. Int J Mod Phys C 16: 281–294.
99. Taylor R (2005) Lévy Fllights. In: Scott A, ed (2005) Encyclopedia of Nonlinear Science. London: Fitzroy-Dearborn.
100. Viswanathan GM, Afanasyev V, Buldyrev SV, Havlin S, da Luz MGE, et al. (2001) Statistical physics of random searches. Brazilian Journal of Physics 31: 102–108.
101. Faure P, Korn H (2001) Is there chaos in the brain? I. Concepts of nonlinear dynamics and methods of investigation. C R Acad Sci III 324: 773–793.
102. Li JS, Huston JP (2002) Non-linear dynamics of operant behavior: a new approach via the extended return map. Rev Neurosci 13: 31–57.

Behavioral/Systems/Cognitive

Flight Initiation and Maintenance Deficits in Flies with Genetically Altered Biogenic Amine Levels

Björn Brembs,[1]* Frauke Christiansen,[1]* Hans Joachim Pflüger,[1] and Carsten Duch[2]

[1]Institute of Biology, Free University of Berlin, 14195 Berlin, Germany, and [2]School of Life Sciences, Arizona State University, Tempe, Arizona 85287

Insect flight is one of the fastest, most intense and most energy-demanding motor behaviors. It is modulated on multiple levels by the biogenic amine octopamine. Within the CNS, octopamine acts directly on the flight central pattern generator, and it affects motivational states. In the periphery, octopamine sensitizes sensory receptors, alters muscle contraction kinetics, and enhances flight muscle glycolysis. This study addresses the roles for octopamine and its precursor tyramine in flight behavior by genetic and pharmacological manipulation in *Drosophila*. Octopamine is not the natural signal for flight initiation because flies lacking octopamine [tyramine-β-hydroxylase (TβH) null mutants] can fly. However, they show profound differences with respect to flight initiation and flight maintenance compared with wild-type controls. The morphology, kinematics, and development of the flight machinery are not impaired in TβH mutants because wing-beat frequencies and amplitudes, flight muscle structure, and overall dendritic structure of flight motoneurons are unaffected in TβH mutants. Accordingly, the flight behavior phenotypes can be rescued acutely in adult flies. Flight deficits are rescued by substituting octopamine but also by blocking the receptors for tyramine, which is enriched in TβH mutants. Conversely, ablating all neurons containing octopamine or tyramine phenocopies TβH mutants. Therefore, both octopamine and tyramine systems are simultaneously involved in regulating flight initiation and maintenance. Different sets of rescue experiments indicate different sites of action for both amines. These findings are consistent with a complex system of multiple amines orchestrating the control of motor behaviors on multiple levels rather than single amines eliciting single behaviors.

Key words: **octopamine; *Drosophila*; tyramine; motor behavior; modulation; invertebrate**

Introduction

How are rhythmical motor behaviors initiated, maintained, and terminated? For many years, neuroscientists have debated whether motor behaviors were produced by chains of reflexes or by intrinsically oscillating central networks. Pioneering work on locust flight set the stage for today's well accepted concept of central pattern generation by demonstrating that rhythmic motor output could be induced by nonrhythmical stimulation of the nerve cord without sensory feedback (Wilson, 1961, 1966; Wilson and Wyman, 1965; Edwards, 2006). The underlying networks are central pattern generators (CPGs), which are found at the heart of motor networks in all animals (Kiehn and Kullander, 2004; Grillner et al., 2005; Marder et al., 2005).

Neuromodulators play a major role in activating and modifying CPG activity (Marder and Bucher, 2001). The central release of specific neuromodulators or mixtures of different modulators can initiate distinct motor patterns (Nusbaum et al., 2001). Pioneering studies in locusts have demonstrated that microinjection of the biogenic amine octopamine (OA) into distinct neuropil regions elicits either walking or flight motor patterns in isolated ventral nerve cords (Sombati and Hoyle, 1984). This has led to the "orchestration hypothesis" (Hoyle, 1985) assuming that neuromodulator release into specific neuropils configures distinct neural assemblies to produce coordinated network activity. Monoamines have also been assigned to aggression, motivation, and mood in vertebrates and invertebrates (Baier et al., 2002; Kravitz and Huber, 2003; Stevenson et al., 2005; Popova, 2006). Furthermore, specific cognitive functions have been assigned to monoamine codes, such as that in flies OA mediates appetitive learning but dopamine mediates aversive learning (Schwaerzel et al., 2003; Riemensperger et al., 2005). In mammals, dysfunctions in monoamine neurotransmission are implicated in neurological disorders, including Parkinson's disease, schizophrenia, anxiety, and depression (Kobayashi, 2001; Taylor et al., 2005).

However, recent work from areas as diverse as Parkinson's disease (Scholtissen et al., 2006) and *Drosophila* larval motor behavior suggests that the chemical codes producing specific motor behavior outputs are bouquets of different amines rather than single ones (Saraswati et al., 2004; Fox et al., 2006). This study

Received June 14, 2007; revised Aug. 28, 2007; accepted Aug. 29, 2007.

This work was supported by the German Science Foundation (C.D., H.J.P.). We are grateful for the help of Fernando Vonhoff with our behavioral experiments. We thank Drs. R. B. Levine (University of Arizona, Tucson, AZ) and J. A. Mustard (Arizona State University, Tempe, AZ) for many critical comments on this manuscript and Marinus de Bruyne and Wernher Fouquet for discussions concerning fly genetics.

*B.B. and F.C. contributed equally to this work.

B.B. helped with experiments and contributed to the design of the study and the writing of the manuscript. F.C. conducted most experiments and contributed to the design of the study. H.J.P. contributed funding and intellectual exchange. C.D. participated in some experiments and contributed to the design of the study and the writing of this manuscript.

Correspondence should be addressed to Carsten Duch, School of Life Sciences, Arizona State University, Tempe, AZ 85287. E-mail: carsten.duch@asu.edu.

DOI:10.1523/JNEUROSCI.2704-07.2007

 0270-6474/07/2711122-10$15.00/0

Table 1. Genotypes and sources of flies

Strains	Genotypes	Source
w^+	+;+;+;+	Dr. H. Scholz, University of Wuerzburg, Wuerzburg, Germany
TβH^{nM18}	TβH^{nM18}/FM7c;+;+;+	Monastirioti et al., 1996
TβH^{nM18} hsp–TβH	w$^-$TβH^{nM18}/FM6;+;P{hsp–TβH};+	Schwaerzel et al., 2003
dTdc2–Gal4	w^{1118};P{Tdc2–Gal4};+;+	Cole et al., 2005
UAS–reaper	w^{1118};+;P{w^{+mc}=UAS–reaper}/ TM3 Sb;+	*Drosophila* Stock Center, Indiana University, Bloomington, IN
w^{1118}	w^{1118};+;+;+	
UAS–2xeGFP	w$^-$;+;P{w^{+mC}=UAS–2xEGFP};+	Halfon et al., 2002

tests this hypothesis by genetic and pharmacological dissection of flight behavior in *Drosophila*. For >20 years, OA has been assigned as the sole modulator controlling insect flight. In contrast, we demonstrate that flight is controlled by the combined action of OA and tyramine (TA). OA and TA are decarboxylation products of the amino acid tyrosine, with TA as the biological precursor of OA. In insect flight systems, OA assumes a variety of physiological roles affecting central neuron excitability (Ramirez and Pearson, 1991), synaptic transmission (Evans and O'Shea, 1979; Leitch et al., 2003), sensory sensitivity (Matheson, 1997), hormone release (Orchard et al., 1993), and muscle metabolism (Mentel et al., 2003). Almost every organ is equipped with OA receptors (Roeder, 1999). TA receptors have been cloned recently in many insect species (Blenau and Baumann, 2003), and physiological functions for TA have been demonstrated (McClung and Hirsh, 1999; Nagaya et al., 2002). The multiple possible levels of OA and TA action on *Drosophila* flight behavior are discussed.

Materials and Methods

Animals

Drosophila melanogaster flies were kept in standard 68 ml vials with cotton stoppers on a yeast–syrup–cornmeal–agar diet at 25°C and 50–60% humidity with a 12 h light/dark regimen. Flies were used for experiments 3–5 d after eclosion. Various strains were used for the experiments (Table 1).

TβH-lines. TβH^{nM18} flies have a null mutation at the tyramine-β-hydroxylase (TβH) locus. The phenotype includes an approximately eightfold increase in tyramine concentration and completely lacks OA (Monastirioti et al., 1996). The strain exhibits female sterility, caused by their inability to lay eggs. Otherwise, the flies appear normal, without dramatic effects on their behavior or lifespan. Because the original TβH^{M18} stock (Monastirioti et al., 1996) carries an additional mutation in the *white* (*w*) gene, the mutant and control stocks from Schwaerzel et al. (2003) were used, as mutations in the white gene might cause unspecific phenotypic effects. The octopamine mutants are recombinant flies with the w^+ allele, and the corresponding nonrecombinant w^+ lines serve as controls (Schwaerzel et al., 2003). Flies of the TβH^{nM18} hsp–TβH strain contain the TβH cDNA under control of the heat-shock protein 70 (HSP70) promoter in the TβH mutant background, making OA synthesis inducible by heat shock (HS) (Schwaerzel et al., 2003).

Gal4 driver lines. The *Drosophila* tyrosine decarboxylase 2 (dTdc2)–galactosidase-4 (Gal4) driver is expressed in clusters of neurons throughout brain and nerve cord. The gene encoding the neuronal enzyme tyrosine decarboxylase (TDC) was identified recently, and the coding section of the yeast GAL4 gene was inserted into it, immediately before the coding start (Cole et al., 2005). We made use of this genetic tool, driving the apoptosis-inducing construct upstream activating sequence (UAS)–reaper and the construct for the enhanced green fluorescent protein (UAS–2xeGFP).

Reporter strains. The cell death gene reaper (White et al., 1994) acts dominantly to kill cells in which it is expressed. Because it has been incorporated into a UAS vector (Zhou et al., 1997), cell-specific ablation can be accomplished efficiently and accurately. The F_1 transheterozygote offspring of the dTdc2–Gal4 × UAS–reaper cross served as the experimental strain. Parent dTdc2–Gal4 and UAS–reaper strains were used as controls. The white-eyed w^{1118} strain was also chosen as control line, because it is the original nonrecombinant line from which the dTdc2–Gal4 and the UAS–reaper strains have been created. dTdc2–Gal4 and UAS–reaper were backcrossed with white, and the progeny was used as heterozygous control. For visualization of octopaminergic and tyraminergic cells, dTdc2–Gal4 virgins were crossed with UAS–2xeGFP (two times enhanced green fluorescent protein) (Halfon et al., 2002) males.

Treatments for behavioral rescue experiments

Octopamine. Flies were raised on OA-containing medium. To obtain an OA (O0250; Sigma, St. Louis, MO) concentration of 10 mg/ml, each vial containing 15 ml of freshly prepared standard food was supplemented with 150 mg of octopamine diluted in 900 μl of distilled water. The OA solution was added while the food was still liquid but at a temperature below 50°C. Distilled water without OA (also 900 μl) was added to control vials. Four-day-old flies were transferred to the vials for oviposition and removed after 24 h. The progeny was raised on the OA-supplemented food and used for experiments later.

Yohimbine. To feed yohimbine (YH) (Y3125; Sigma), a 5% sucrose (S1888; Sigma) solution with or without yohimbine added (10 mg/ml) was pipetted onto five pieces of filter paper in cylindrical vials before transferring 10–20 mutants into the vials. After 1–2 h, the animals were singled out and prepared for testing.

Heat shock. Flies (TβH^{nM18} hsp–TβH) were kept at 37°C for 45 min twice with a 6 h interval and were then allowed to recover for 12 h before experiments.

Behavioral testing

Three- to 5-d-old male flies were briefly immobilized by cold anesthesia and glued [clear glass adhesive (Duro; Pacer Technology, Rancho Cucamonga, CA)] with head and thorax to a triangle-shaped copper hook (0.02 mm diameter). Adhesion was achieved by exposure to UV light for 10 s. The animals are then kept individually in small chambers containing a few grains of sucrose until testing (1–5 h).

The fly, glued to the hook as described above, was attached to the experimental setup via a clamp to accomplish stationary flight. For observation, the fly was illuminated from behind and above (150 W, 15 V; Schott, Elmsford, NY) and fixed in front of a polystyrene panel. Additionally, it was shielded by another polystyrene panel from the experimenter. Tarsal contact with a bead of polystyrene prevented flight initiation before the experiment started. A digital high-speed camera (1000 pictures per second; Motion Scope; Redlake Imaging, Morgan Hill, CA) was positioned behind the test animal. To initiate flight, the polystyrene bead was removed, and the fly was gently aspirated. The time until the fly ceased flying was recorded (initial flight). The fly was aspirated as a stimulation to fly, each time it stopped flying. When no flight reaction was shown after three consecutive stimulations, the experiment was completed and the total flight time was recorded (extended flight). Every stimulus after the first one, to which the fly showed a response, was recorded. Each fly was filmed during the first few seconds of flight, and the recordings were saved on a personal computer for later analysis. The person scoring the flight time was unaware of the treatment group of the animal. All animals were included in the study, including those that did not show any flight behavior.

Neuroanatomical stainings

Immunocytochemistry. For immunohistochemical stainings of *Drosophila* CNS with GFP antibody (Ab), fly CNS was removed in saline. After fixation for 1 h in 4% paraformaldehyde (PFA) (10 ml of PBS plus 0.4 g of PFA, pH 7.4), the CNS was treated with a mixture of enzymes (colla-

genase/dispase, 1 mg/ml each) for 1 min to ensure better penetration of antibodies (Abs) into the tissue and then washed in PBS (0.1 M) overnight at 4°C. Preparations were then washed six times for 30 min in 0.5% Triton X-100 in PBS (PBSTx), again to increase the penetration of Ab into the tissue. Subsequently, the CNS was placed for 2 d in a 1:200 dilution of the anti-GFP primary Ab mouse serum in 0.3% PBSTx at 4°C. They were then rinsed eight times for 15 min in PBS and then incubated at 4°C overnight in a 1:500 dilution of the secondary Ab serum that was coupled to a fluorescent dye [anti-mouse cyanine 2 (Cy2)] in PBS. After rinsing the preparations eight times for 15 min in PBS, they were dehydrated in an ascending ethanol series (50, 70, 90, and 100%, 10 min each) and then transferred to a microscope slide and cleared in methylsalicylate. For immunohistochemical stainings of *Drosophila* CNS for presynaptic active zones with bruchpilot antibody (Wagh et al., 2006) (gift from E. Buchner, University of Wuerzburg, Wuerzburg, Germany), the same protocol was followed with the exception that the primary Ab was diluted 1:100 in 0.3% PBSTx.

Phalloidin stainings. Flies were opened via a dorsal longitudinal cut in saline and then fixed in 4% PFA. After 1 h, they were transferred into PBS, and flight muscles were removed and washed three times for 1 h in 0.5% PBSTx. After treatment with 2 μl/ml Oregon Green phalloidin, 0.3% PBSTx for 36 h, the muscles were washed six times for 15 min in PBS and finally embedded in glycerin on a microscope slide.

Confocal microscopy. The preparations were viewed under a Leica (Bensheim, Germany) SP2 confocal laser-scanning microscope with 40× oil immersion objective. Stacks of optical sections (0.5 μm) were acquired. Both Cy2 and Oregon Green phalloidin were excited with an argon laser at 488 nm, and emitted light was detected between 500 and 530 nm.

Data analysis

Wing-beat amplitude. For wing-beat amplitude measurements, Redlake Imaging MotionScope software (DEL Imaging Systems, Cheshire, CT) was used to capture the first 100 frames. After image inversion, the image stacks were imported into AMIRA software (TGS, San Diego, CA) for overlaying of all frames (projection view) and then measuring wing angles using the angle-measuring tool.

Wing-beat frequency. To measure the wing-beat frequency, the number of frames per 10 wing beats was counted, starting from frame 1, 100 and 300 in each sequence, and subsequently the mean was calculated.

Sarcomere length. For sarcomere-length survey, the images of phalloidin-stained muscles were imported into AMIRA software, and sarcomeres were measured with the line-measuring tool. For each animal, the lengths of 31–41 sarcomeres were measured.

Flight time per stimulation. To calculate flight time per stimulation, the total flight time was divided by the number of stimulations, including the initial one.

Statistics. The flight data approximately conformed to a Poisson distribution, and hence nonparametric tests were used. For comparison of more than two groups, a Kruskal–Wallis ANOVA was used to test the hypothesis that the samples were drawn from the same population. When differences between the samples occurred, Mann–Whitney *U* tests were performed for planned comparisons of two samples. Two groups were always compared with a Mann–Whitney *U* test. To display the measurements, box-and-whisker plots were chosen, and medians were used as central values. Boxes included the medial 25–75%, and, because the data show many extreme scores, the whiskers included 15–85% of the data values. Outliers were not shown. Significant differences were accepted at $p < 0.05$.

A full rescue is scored when the rescue group differs significantly from the mutant but not from the wild-type control. For a partial rescue, the rescue line must either differ significantly from both mutant and wild type or not differ from both. No rescue is achieved when no significant difference is obtained between the mutant flies and the rescue line and a significant difference remains for the wild-type controls.

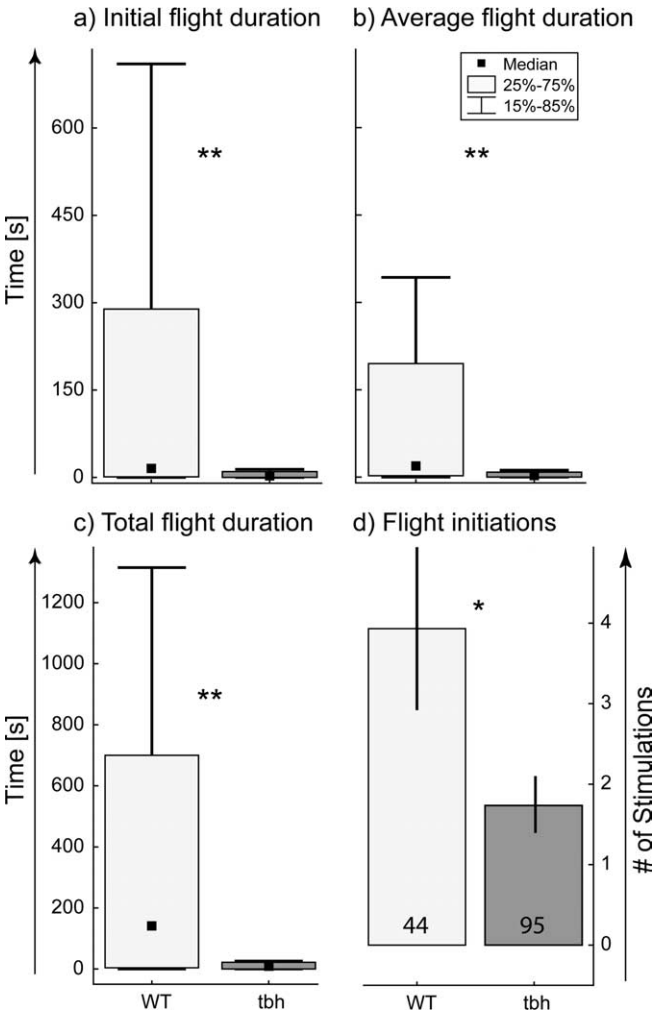


Figure 1. Comparison of flight initiation and maintenance between TβH mutant and wild-type flies. For ***a–c***, the black squares indicate the median, the boxes signify the 25 and the 75 percentiles, and the error bars range from the 15 to the 85 percentiles. ***a*** shows the flight duration until the first stop for wild-type (WT; light gray bar) and TβH null mutant (tbh; dark gray bar) flies. ***b*** indicates the duration of all flight bouts for wild-type and TβH flies. ***c*** shows the total flight duration for wild-type and TβH flies. ***d*** shows the mean number of stimuli to which wild-type and TβH mutant flies responded with flight bouts before they did not respond to three consecutive stimuli (error bars are SEMs). The number of animals per group is indicated in the bars. $^*p < 0.05$, $^{**}p < 0.01$, Mann–Whitney *U* test.

Results

Flight initiation and maintenance deficits in flies lacking octopamine

There currently is only one viable strain lacking OA, a null mutant in the TβH gene, TβH^{nM18} (Monastirioti et al., 1996). Mutants lacking OA are able to fly, clearly demonstrating that OA is not required for flight initiation. However, TβH^{nM18} mutants show a drastic decrease in the initial flight duration (Fig. 1*a*), in all subsequent flight episodes [i.e., average flight duration per stimulation (Fig. 1*b*, Average flight duration)] and thus also in total flight duration (Fig. 1*c*, Total flight duration). Moreover, the mutants resume flight less often after stimulation compared with control animals (Fig. 1*d*, Flight initiations). Therefore, TβH^{nM18} mutants take off significantly less often in response to wind stimuli than wild-type controls (Fig. 1*d*), and, once airborne, they fly for significantly shorter durations (Fig. 1*a–c*).

A number of flight motor system parameters do not differ between mutants and wild type, suggesting that the basic func-

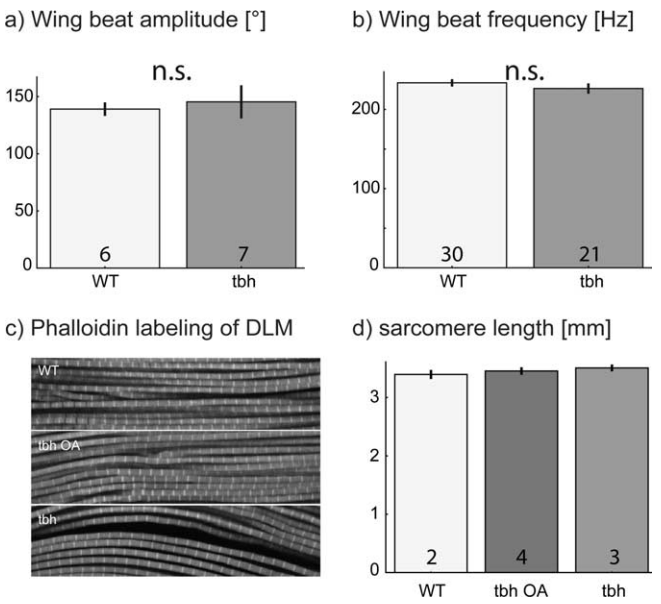


Figure 2. The development of the flight system is not impaired in TβH mutant flies. ***a*** shows the mean wing-beat amplitudes for wild-type (WT; light gray bar) and TβH mutant (tbh; dark gray bar) flies. ***b*** shows the mean wing-beat frequencies for wild-type (WT; light gray bar) and TβH mutant (tbh; dark gray bar) flies. ***c*** shows representative fields of view of DLM flight muscle fibers with phalloidin-labeled actin bands for wild-type (WT), TβH mutant (tbh), and TβH mutant flies that were fed with octopamine (tbh OA). ***d*** shows the mean sarcomere lengths for the three groups shown in ***c***. Numbers in bars indicate numbers of animals. Error bars are SEMs. n.s., Not significant.

tion and morphology of the flight apparatus is unaffected in TβH^{nM18} mutants. With regard to motor output, wing-beat amplitudes (Fig. 2*a*) and wing-beat frequencies are similar in TβH^{nM18} mutants and wild-type controls (Fig. 2*b*). On the muscular level, sarcomere length of the dorsal longitudinal flight muscle (DLM) flight is not affected in TβH^{nM18} mutants (Fig. 2*c*,*d*). Figure 2, *c* and *d*, includes a third group of flies, TβH^{nM18} mutants that were fed with octopamine to rescue the flight behavior phonotype (see below). Sarcomere lengths are similar in wild type, TβH^{nM18} mutants, and TβH^{nM18} mutants rescued by feeding octopamine. Within the CNS, the overall morphology of the DLM motoneurons MN1–MN5 appears similar between wild-type controls and TβH^{nM18} mutants as revealed by dye backfilling from the DLM flight muscle (data not shown). Consequently, the observed changes in flight behavior may be attributable to the acute changes in the titers of OA and TA (lack of OA and increase in TA) rather than to developmental defects. However, there may be differences in the number and strength of synaptic inputs or in the fine branching structure of flight motoneurons and interneurons, which were not subjected to this study. To further test whether the acute lack of OA in adults was a main cause for the observed flight behavior deficits, we conducted a number of rescue experiments.

Manipulating octopamine and tyramine rescues flight initiation and maintenance

Rescuing the phenotype in TβH^{nM18} mutants is not a trivial task, because these flies not only lack OA but also show an eightfold increase in the concentration of the OA precursor TA. To adequately address this issue, we designed rescue experiments combining pharmacological and genetic techniques. For clarity, the tyramine and octopamine biosynthesis pathway is shown schematically in Figure 3*e*; genetic or pharmacological knockdowns as used throughout this study are indicated in light gray, and genetic or pharmacological rescues are indicated in dark gray. To oppose the effects of increased TA concentration, we fed the flies the selective competitive α2-adrenergic receptor antagonist YH, which has been demonstrated to block *Drosophila* tyramine receptors (TARs) (Arakawa et al., 1990; Saudou et al., 1990). To increase OA concentration in TβH^{nM18} mutants, we either fed the flies OA or induced TβH expression in all cells via an HS-inducible TβH transgene in the TβH null mutant genetic background. The following four permutations were tested as experimental groups: (1) TβH^{nM18}; hsp–TβH + HS, (2) TβH^{nM18}; hsp–TβH + HS + YH, (3) TβH^{nM18}; hsp–TβH + YH, and (4) TβH^{nM18} + OA. The three negative control groups were TβH null mutants, TβH null mutant with a heat-shock-inducible TβH transgene kept at normal temperature, and TβH null mutants without inducible TβH transgene were exposed to the heat shock (TβH^{nM18}, TβH^{nM18} hsp–TβH, and TβH^{nM18} + HS). The three control groups do not differ in any of the flight behavior parameters investigated (data not shown), and their data were thus pooled. The w^+ strain serves as positive control (for strain genotype, see Materials and Methods).

For the duration of the initial flight phase, we obtained a full rescue in all four experimental groups (Fig. 3*a*, see inset for comparison of medians only). Feeding YH and treating with HS in the same flies (HS + YH) yields the best rescue (median of 9; $p < 0.001$ compared with TβH flies, $p = 0.464$ compared with wild-type flies) followed by feeding YH only (median of 6; $p = 0.001$ compared with TβH flies, $p = 0.284$ compared with wild-type flies). Next are feeding OA (median of 8; $p = 0.005$ compared with TβH flies, $p = 0.1$ compared with wild-type flies) and HS only (median = 4; $p = 0.013$ compared with TβH flies, $p = 0.169$ compared with wild-type flies). In summary, blocking TA action pharmacologically, replacing OA genetically or pharmacologically, or combining TA and OA manipulations rescues the TβH phenotype with respect to the duration of the initial flight bout.

Average flight duration per stimulation is at least partially rescued in all experimental groups (Fig. 3*b*, see inset for comparison of medians). A full rescue is obtained only by feeding YH alone (median of 4; $p < 0.001$ compared with TβH flies, $p = 0.114$ compared with wild-type flies). Partial rescues can be achieved with HS + YH (median of 7; $p < 0.001$ compared with TβH flies, $p = 0.047$ compared with wild-type flies), with HS (median of 2; $p = 0.025$ compared with TβH flies, $p = 0.032$ compared with wild-type flies), and by feeding OA (median of 3; $p = 0.025$ compared with TβH flies, $p = 0.015$ compared with wild-type flies). In summary, a full rescue of the average flight duration in multiple subsequent flight bouts is achieved only by blocking TA receptors but not by replacing OA either genetically or pharmacologically.

The duration of total flight (Fig. 3*c*) can be fully rescued by HS + YH (median of 72; $p < 0.001$ compared with TβH flies, $p = 0.259$ compared with wild-type flies), by only feeding YH (median of 40; $p < 0.001$ compared with TβH flies, $p = 0.441$ compared with wild-type flies), and by HS (median of 30; $p = 0.002$ compared with TβH flies, $p = 0.076$ compared with wild-type flies) but not by supplementing OA alone (median of 11; $p = 0.163$ compared with TβH flies, $p = 0.005$ compared with wild-type flies). Total flight duration is the product of the number of flight initiations times the average time of the flight bouts. The average time of the flight bouts is partially rescued by feeding OA

(Fig. 3*b*), but the number of responses (flight initiations) is not rescued by feeding OA to TβH flies (Fig. 3*d*).

The responsiveness to stimulation (Fig. 3*d*) was fully rescued by feeding YH (median of 10; $p < 0.001$ compared with TβH flies, $p = 0.083$ compared with wild-type flies) and by HS (median of 8,1; $p = 0.021$ compared with TβH flies, $p = 0.599$ compared with wild-type flies). Feeding OA only did not rescue this phenotype ($p = 0.994$ over TβH flies, $p = 0.053$ over wild-type flies) but even caused a slight but nonsignificant decrease in the responsiveness to stimulation. HS + YH-treated animals responded to stimulation even more often than wild-type flies (median of 9.3; $p < 0.001$ compared with TβH flies, $p = 0.028$ compared with wild-type flies).

This complex set of full and partial rescues depending on OA and TA manipulation demonstrates that flight behavior depends on OA and on TA. One possibility is that OA and TA each act on different aspects of the flight machinery, such as sensory sensitivity, muscle metabolism, or CPG activation. Alternatively, OA and TA might act antagonistically on similar aspects of motor behavior, and thus, the absolute levels of one modulator are not important, but the relative levels of both modulators influence flight behavior. In a first test of the latter hypothesis, we ablated all neurons synthesizing TA from tyrosine by expressing the apoptosis-inducing gene *reaper* under control of the dTdc2 promotor (for details, see Materials and Methods). The dTdc2 gene codes for the neural version of two TDC enzymes converting tyrosine to TA.

Because TA is the precursor of OA, dTdc2 expresses in all neurons containing TA or OA, as can be visualized by expressing eGFP under the control of dTdc2 and enhancing the eGFP signal by anti-GFP immunocytochemistry (Fig. 4*a*). Cell bodies of dTdc2 neurons are located in the midlines of each thoracic and each abdominal neuromere, bilateral symmetric processes of efferent unpaired median neurons can clearly be seen, and a large number of finer aminergic processes with numerous varicosity-like structures can be visualized within the CNS (Fig. 4*a*).

Expressing the apoptosis signal *reaper* under the control of dTdc2 causes a complete and specific ablation of TA- and OA-containing neurons (Fig. 4*b*,*c*). This genetic ablation of all neurons releasing TA or OA also leads to a profound decrease in all four behavioral parameters studied compared with control strains (Fig. 5). The genetic controls were parent dTdc2–

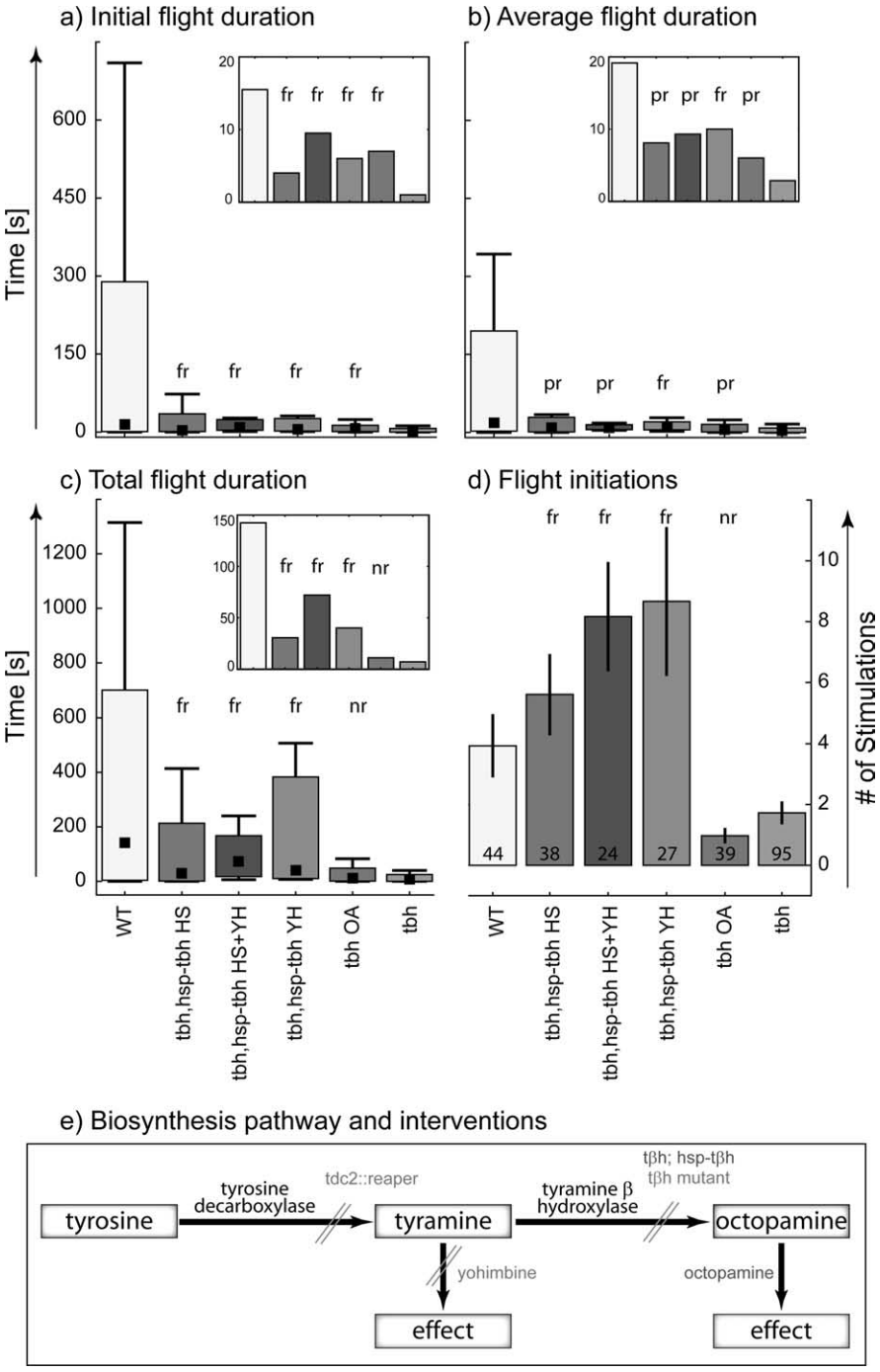


Figure 3. Different types of rescues of the TβH^{nM18} caused flight behavior phenotypes. For ***a–c***, the black squares indicate the median, the boxes signify the 25 and the 75 percentiles, and the error bars range from the 15 to the 85 percentiles. To allow for a better between-group comparison, insets in ***a*** to ***c*** depict bar graphs of the respective medians at a higher *y*-axis resolution. ***a*** shows the duration of the initial flight bout for each experimental group, ***b*** shows the average duration of a flight bout for each group, ***c*** shows the total flight duration, and ***d*** shows the number of stimuli to which the flies responded with flight before they did not respond to three consecutive stimuli. fr, Full rescue; pr, partial rescue; nr, no rescue (for definition, see Materials and Methods). The experimental groups were wild-type flies (WT), a genetic rescue in which TβH expression in TβH mutant flies was induced in all cells via a heat-shock inducible TβH transgene in the TβH null mutant genetic background (tbh, hsp–tbh HS), a combined genetic and pharmacological rescue in which TβH expression was induced via a heat shock and in which the flies were also fed the tyramine receptor blocker yohimbine (tbh, hsp–tbh HS + YH), a pharmacological rescue in which TβH mutant flies containing the inducible TβH transgene received no heat shock but were fed yohimbine (tbh, hsp–tbh YH), a pharmacological rescue in which TβH mutant flies were fed octopamine (tbh OA), and TβH mutant flies (tbh). ***e*** shows the biosynthesis pathway of tyramine and octopamine from tyrosine. Genetic and pharmacological blocks are depicted in light gray. TA synthesis is blocked by killing all cells containing tyrosine decarboxylase by expressing reaper. OA synthesis is blocked in tyramine hydroxylase null mutants (TβH^{nM18}). TARs are blocked by yohimbine. Rescues are depicted in dark gray. Octopamine levels were increased by either expressing tyramine hydroxylase under the control of a heat shock promoter or by feeding OA.

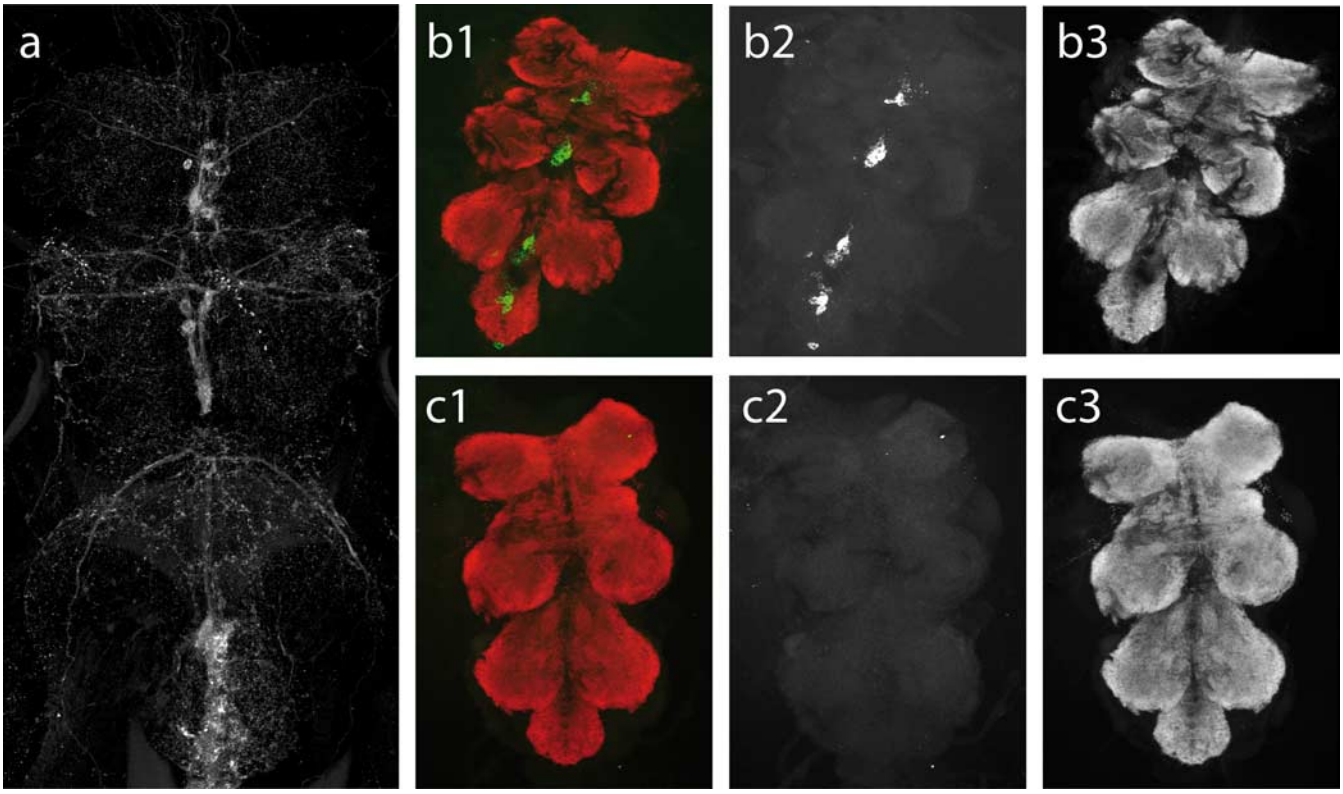


Figure 4. Genetic ablation of all tyraminergic and octopaminergic neurons. ***a***, Visualization of all tyraminergic and octopaminergic neurons in the thoracic and abdominal ventral nerve cord by expressing 2xeGFP under the control of Tdc2 and enhancing the signal by anti-GFP immunocytochemistry. To test the effectiveness of neuron ablation by targeted ectopic expression of the cell death gene reaper, animals expressing either only GFP or GFP together with reaper were subjected to standard immunohistochemistry. Animals expressing only GFP reveal the expression pattern typical of Tdc2 neurons. ***b1*** shows double labels of the ventral nerve cord for Tdc2 neurons labeled by targeted expression of eGFP (green) and all synapses labeled with bruchpilot antibody Nc82 (Kittel et al., 2006) (red) to visualize presynaptic active zones in the neuropil regions. ***b2*** and ***b3*** show the Tdc2 and the Nc82 signal separately as grayscale images. ***b1–b3*** show a ventral nerve cord from heterozygous progeny of dTdc2–Gal4 crossed with y w P{w $^{+mC}$ = UAS–2xEGFP}. ***c***, Gal4-driven apoptosis was induced by crossing dTdc2–Gal4 with w;; P{UAS-rpr}/TM3 Sb, and eGFP was from y w P{w $^{+mC}$ = UAS–2xEGFP}. No GFP expression can be detected in animals with targeted expression of both GFP and reaper to these OA/TA cells (***c1***), but Nc82 immunostaining appears unaffected in these animals (***c3***), demonstrating effective and specific ablation.

Gal4 and UAS–reaper strains. The white-eyed w^{1118} strain was also chosen as control line, because it is the original nonrecombinant line from which the dTdc2–Gal4 and the UAS–reaper strains have been created. dTdc2–Gal4 and UAS–reaper flies were backcrossed with white flies, and the progeny were used as heterozygous controls. The three control groups did not differ in flight behavior (data not shown), and their data were pooled (Fig. 5). Similar to knocking out OA only in TβH^{nM18} mutants (Fig. 1), ablating all TA and OA neurons drastically decreased the initial flight duration (Fig. 5*a*), the flight duration per stimulation (Fig. 5*b*), and extended flight (Fig. 5*c*, Total flight duration). Moreover, the mutants resumed flight less often after stimulation compared with control animals (Fig. 5*d*). However, it is noteworthy that flies with all TA- and OA-containing neurons ablated were still able to fly, and wing-beat frequencies were normal. In summary, in flies without TA- or OA-containing neurons, flight initiation and maintenance are affected in a similar manner to flies lacking OA but having increased TA levels.

At first glance, it appears contradictory that TβH^{nM18} mutants can be rescued by blocking TA receptors, but flies without OA and without TA show behavioral phenotypes similar to TβH^{nM18} mutants. This result clearly opposes the interpretation that OA and TA simply act antagonistically on the same targets, but it might be explained by dose effects and different sites of action (see Discussion). However, we further tested the effects of TA on flight behavior in flies with normal OA and TA levels by pharmacological block of TA action.

We compared initial flight (Fig. 6*a*), mean flight bout duration (Fig. 6*b*), total flight duration (Fig. 6*c*), and the number of stimulations causing flight (Fig. 6*d*) in wild-type flies that were fed with yohimbine and wild-type controls that were fed with sucrose solution only. Feeding yohimbine yields the most effective rescues of flight initiation and maintenance in TβH^{nM18} mutants (Fig. 3). However, none of these flight parameters is different among sucrose-fed and yohimbine-fed wild-type flies (Fig. 6). Consequently, flight initiation and maintenance do not depend strictly on the relative levels of OA and TA but are affected by some concerted interaction of both amines. Depleting OA and increasing TA impairs flight motor behavior, as does ablation of all OA- and TA-containing neurons. In OA-depleted flies with increased TA, flight initiation and maintenance can be rescued either by restoring OA levels or blocking TA action. In contrast, blocking TA action in flies with normal OA and TA levels does not affect any of the flight motor behavior parameters measured in this study.

Discussion

OA is not required for flight initiation

Flies lacking OA and having increased TA levels (TβH null mutants) show a profound decrease in flight initiation and maintenance compared with wild-type controls. Five lines of evidence suggest that morphology, kinematics, and development of the flight machinery are not impaired in TβH mutants: (1) wing-beat frequencies, (2) wing-beat amplitudes,

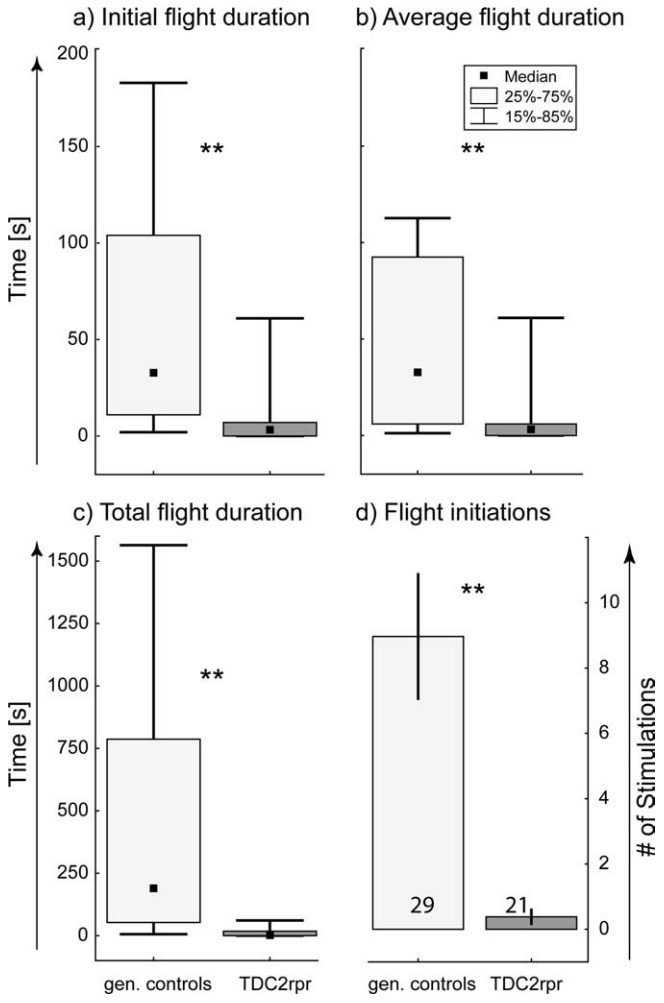


Figure 5. Genetic ablation of all tyraminergic and octopaminergic neurons decreases flight initiation and maintenance. For ***a–c***, the black squares indicate the median, the boxes signify the 25 and the 75 percentiles, and the error bars range from the 15 to the 85 percentiles. ***a*** shows the flight duration until the first stop in control flies (gen. controls; light gray bar) and for flies expressing reaper under the control of TDC2 (TDC2rpr; dark gray bar). ***b*** indicates the mean duration of all flight bouts for control and TDC2rpr flies. ***c*** shows the total flight duration for control and TDC2rpr flies. ***d*** shows the mean number of stimuli to which control and TDC2rpr responded with flight bouts before they did not respond to three consecutive stimuli (error bars are SEMs). $^{**}p < 0.01$, Mann–Whitney *U* test.

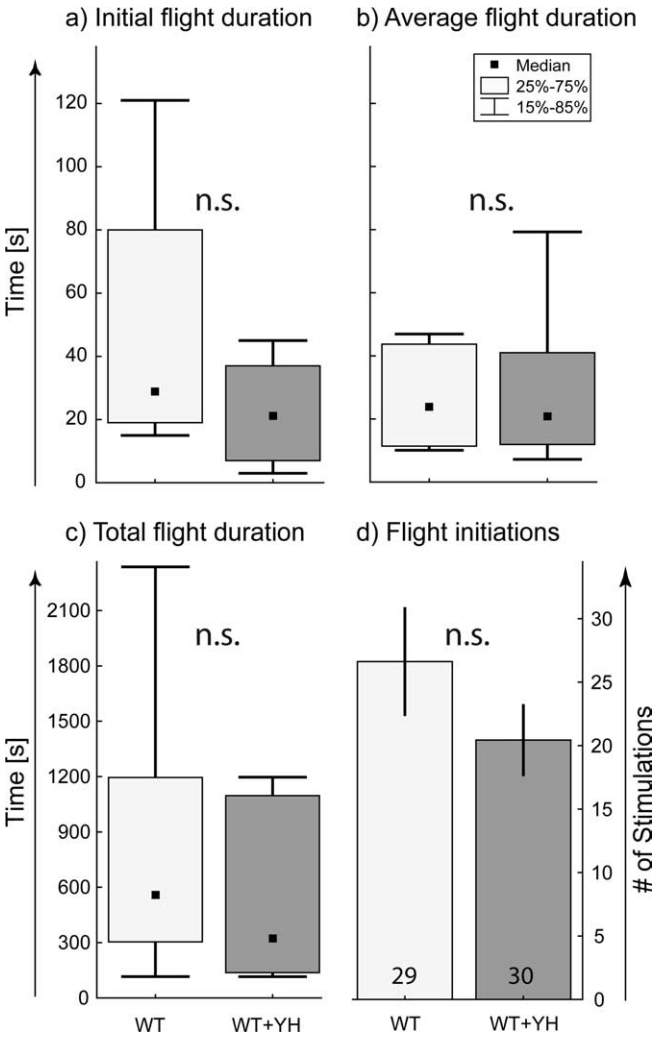


Figure 6. Blocking TA receptors in wild-type flies does not affect flight behavior. For ***a–c***, the black squares indicate the median, the boxes signify the 25 and the 75 percentiles, and the error bars range from the 15 to the 85 percentiles. ***a*** shows the flight duration until the first stop in control wild-type flies fed with sucrose (WT; light gray bar) and for wild-type flies fed with the TA receptor blocker yohimbine (WT + YH; dark gray bar). ***b*** indicates the average duration of flight bouts for WT control and WT + YH flies. ***c*** shows the total flight duration for WT control and WT + YH flies. ***d*** shows the mean number of stimuli to which WT control and WT + YH responded with flight bouts before they did not respond to three consecutive stimuli (error bars are SEMs). n.s. indicates that no significant differences were found, Mann–Whitney *U* test.

(3) flight muscle structure (length of myofibrils), and (4) the number and overall dendritic structure of flight motoneurons are unaffected in TβH mutants, and (5) the behavioral phenotype can acutely be rescued in adult flies. Although acute application of OA is sufficient to elicit flight in a number of different insect preparations (Sombati and Hoyle, 1984; Claassen and Kammer, 1986; Stevenson and Kutsch, 1987; Duch and Pflueger, 1999), OA is not necessary for the initiation of flight in *Drosophila* but modulates flight initiation and maintenance. Even flies without any OA/TA-containing neurons can fly. Therefore, OA is either not a necessary natural signal for flight initiation or *Drosophila* flight initiation is a unique case.

Concerted action of OA and TA on flight behavior

A novel finding is that flies lacking OA and with TARs blocked show wild-type-like flight behavior. It is important to note that the TβH phenotype comprises OA knock-out plus eight-fold increased TA levels. Pharmacological blockade of TARs yields the most efficient rescue of the TβH mutants, even outscoring replacement of OA by heat-shock plus TAR blockade. However, blocking TARs in wild-type flies does not increase flight initiation or maintenance. This indicates that TA inhibits flight behavior only at abnormally high TA levels. Furthermore, with regard to flight maintenance, the inhibitory effects of TA take place only at low OA levels, because OA replacement without affecting the TA system also yields rescues of the initial and the average flight bout durations. In contrast, the responsiveness to stimulation is rescued best by blocking TA. Therefore, flight initiation is most likely inhibited by high TA levels, regardless of the OA levels. Accordingly, feeding TβH mutants OA does not rescue flight initiation but restoring tyramine-β-hydroxylase activity by heat shock does, because only the latter manipulation decreases the levels of TA

by conversion of TA into OA. Therefore, the most parsimonious interpretation is that OA is necessary for flight maintenance, and TA acts most likely as an inhibitor, especially for flight initiation at high concentrations.

This interpretation is further supported by ablating all OA/TA neurons by expressing the apoptosis factor *reaper* in these cells. Flies without OA/TA neurons show the same massive changes in flight behavior as TβH mutants. Therefore, genetic ablation of all TA/OA-containing neurons does not phenocopy genetic ablation of the OA-producing enzyme paired with pharmacological block of TA action. How can these seemingly contradictory results be explained? Clearly, the pharmacological treatment with yohimbine is effective; it fully rescues the mutant phenotype. The ablation of the OA/TA neurons is equally effective, ruling out methodological flaws. However, yohimbine does most likely not block all TA action, whereas genetic ablation of all TA-containing neurons does. Thus, the action of TA presumably follows a bell-shaped curve, with its presence necessary for normal flight but hindering flight initiation and maintenance at high concentration. OA is required most likely for flight maintenance because feeding it to TβH mutants fully rescues normal flight maintenance. However, OA supplementation in the food might also exert rescuing effects in TβH mutants by downregulating TA via feedback inhibition. In summary, the most compelling explanation for the data are that OA is boosting flight maintenance, low levels of TA are required for flight maintenance and initiation, and inhibitory TA actions fall in place at high TA and low OA levels.

TA as neurotransmitter/modulator

Our finding that OA and TA are involved in regulating flight emphasizes the role of TA as an independent neurotransmitter in invertebrates. Further supporting this role, tyramine-like immunoreactivity has been demonstrated in non-octopaminergic cells of *Caenorhabditis elegans* and locusts (Stevenson and Spoerhase-Eichmann, 1995; Donini and Lange, 2004; Alkema et al., 2005). Moreover, at least one *Drosophila* amine receptor is specific for TA and does not cross-react with OA (Cazzamali et al., 2005). Furthermore, OA and TA receptor distributions in the insect CNS differ considerably from each other [J. Erber (Technical University Berlin, Berlin, Germany), personal communication]. Functionally, exogenous TA increases chloride conductances in *Drosophila* malpighian tubules (Blumenthal, 2003), alters body wall muscle excitatory junction potentials (Kutsukake et al., 2000), and can rescue cocaine sensitization in *Drosophila* (McClung and Hirsh, 1999). In mammals, the physiological roles for trace amines such as TA and OA are mostly unknown, but they have been implicated in a variety of neurological disorders (Branchek and Blackburn, 2003), and receptors specific for TA have been identified (Borowsky et al., 2001). In invertebrates, a role of endogenous TA as an important transmitter/modulator has been shown for *Drosophila* locomotor (Saraswati et al., 2004; this study) and olfactory avoidance (Kutsukake et al., 2000) behavior, as well as for *C. elegans* motor behavior (Alkema et al., 2005).

Sites of OA and TA action

Previous studies suggested that OA acts as a potent, direct stimulator of flight muscle metabolism (Wegener, 1996; Mentel et al., 2003). Accordingly, we expected that especially prolonged flight would be affected in TβH mutants, attributable to insufficient fuel supply. In contrast, all flight parameters are similarly affected in TβH mutants. The initial flight bout duration is decreased ~40 times, and the total flight duration is decreased ~30 times in TβH mutants. Moreover, flight behavior changes in TβH mutants are rescued by blocking TA action alone, leaving OA levels unaltered. This is hard to reconcile with direct effects of OA on flight metabolism and would require independent effects of OA and TA on flight metabolism. These considerations render metabolism unlikely as the site of action for OA. Therefore, amine effects on *Drosophila* flight initiation and maintenance are more likely to be mediated by effects on the nervous system.

Two main OA/TA effects on flight behavior can be observed: maintenance of flight and the probability of initiating flight. In principle, both could be controlled by aminergic action on the CPG and/or on the fly's sensory system. It is well established that OA acts on the CPG in a number of insect species (Sombati and Hoyle, 1984; Claassen and Kammer, 1986; Stevenson and Kutsch, 1987), but central actions of TA are not known. OA has also been reported to increase the responsiveness of flight-associated sensory cells in insects (Ramirez and Orchard, 1990), and TA could conceivably reduce excitability of sensory neurons as *Drosophila* TARs activate chloride currents (Cazzamali et al., 2005).

Motor behavior specificity of combined amine effects

OA and TA have been implicated as agonist and antagonist, respectively, controlling locomotor behavior in *Drosophila* larvae (Saraswati et al., 2004; Fox et al., 2006) and in *C. elegans* (Alkema et al., 2005). This raises the possibility of a general, opponent OA/TA control of locomotor behavior in invertebrates. Our results make it unlikely that OA and TA simply act antagonistically on the same targets because, with regard to flight initiation and maintenance, OA and TA probably have different sites of action and TA effects are important only at high TA and low OA levels. Nevertheless, in some preliminary experiments, we tested whether TβH^{nM18} mutant adults show also walking behavior deficits. Neither the overall motor activity per unit time nor the number of walking bouts differed between wild-type and TβH^{nM18} mutant flies. However, we found a slight but statistically significant reduction in walking speed in TβH^{nM18} mutants (data not shown). These findings indicate that aminergic modulation by OA and TA does not act generally on locomotor performance but specifically affects different aspects of motor behaviors.

In summary, the emerging picture is that, for some motor behaviors, the concerted interaction of specific biogenic amines is more important than the concentration of single amines (Scheiner et al., 2002; Schwaerzel et al., 2003; Saraswati et al., 2004; Alkema et al., 2005; Fox et al., 2006; Fussnecker et al., 2006). The current study is the first to suggest that the antagonistic actions of OA and TA are not a general feature of all invertebrate locomotor behaviors but specifically affect distinct aspects of different motor behaviors. It provides evidence that OA and TA do not simply act antagonistically on the same targets but most likely mediate their effects on motor performance by affecting different targets in a dose-dependent manner. The next steps toward understanding amine function for motor behavior is to determine their sites of action during behavior. One possibility addressing this question is to combine pharmacological and genetic rescues and test immunocytochemically where the OA and TA levels are restored in which rescue procedure, how behavior is affected in these different manipulations, and where the various subtypes of TA and OA receptors are localized. Ultimately, a complete under-

standing of the mechanism by which various modulators interact on different parts of the brain and other tissues to control motor behavior will require a large number of targeted manipulations of each individual circuit component separately.

References

Alkema MJ, Hunter-Ensor M, Ringstad N, Horvitz HR (2005) Tyramine functions independently of octopamine in the *Caenorhabditis elegans* nervous system. Neuron 46:247–260.

Arakawa S, Gocayne JD, McCombie WR, Urquhart DA, Hall LM, Fraser CM, Venter JC (1990) Cloning, localization, and permanent expression of a *Drosophila* tyramine receptor. Neuron 2:342–354.

Baier A, Wittek B, Brembs B (2002) *Drosophila* as a model organism for the neurobiology of aggression. J Exp Biol 205:1233–1240.

Blenau W, Baumann A (2003) Aminergic signal transduction in invertebrates: focus on tyramine and octopamine receptors. Recent Res Dev Neurochem 6:225–240.

Blumenthal EM (2003) Regulation of chloride permeability by endogenously produced tyramine in the *Drosophila* malphigian tubule. Am J Cell Physiol 284:C718–C728.

Borowsky B, Adham N, Jones KA, Raddatz R, Artymyshyn R, Ogozalek KL, Durkin MM, Lakhlani PP, Bonini JA, Pathirana S (2001) Trace amines: identification of a family a mammalian G protein-coupled receptors. Proc Natl Acad Sci USA 98:8866–8971.

Branchek TA, Blackburn TP (2003) Trace amines receptors as targets for novel therapeutics: legend, myth and fact. Curr Opin Pharmacol 3:90–97.

Cazzamali G, Klaerke DA, Grimmelikhuijzen CJP (2005) A new family of insect tyramine receptors. Biochem Biophys Res Comm 2:1189–1196.

Claassen DE, Kammer AE (1986) Effects of octopamine, dopamine, and serotonin on production of flight motor output by thoracic ganglia of *Manduca sexta*. J Neurobiol 17:1–14.

Cole SH, Carney GE, McClung CA, Willard SS, Taylor BJ, Hirsh J (2005) Two functional but noncomplementing *Drosophila* tyrosine decarboxylase genes: distinct roles for neural tyramine and octopamine in female fertility. J Biol Chem 280:14948–14955.

Donini A, Lange AB (2004) Evidence for a possible neurotransmitter/neuromodulator role of tyramine on the locust oviduct. J Insect Physiol 50:351–361.

Duch C, Pflueger HJ (1999) DUM neurons in locust flight: a model system for amine-mediated peripheral adjustments to the requirements of a central motor program. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 184:489–499.

Edwards JS (2006) The central nervous control of insect flight. J Exp Biol 209:4411–4413.

Evans PD, O'Shea M (1979) An octopaminergic neurone modulates neuromuscular transmission in the locust. Nature 270:257–259.

Fox LE, Soll DR, Wu CF (2006) Coordination and modulation of locomotion pattern generators in *Drosophila* larvae: effects of altered biogenic amine levels by the tyramine β hydroxylase mutation. J Neurosci 26:1486–1498.

Fussnecker BL, Smith BH, Mustard JA (2006) Octopamine and tyramine influence the behavioral profile of locomotor activity in the honey bee (*Apis mellifera*). J Insect Physiol 52:1083–1092.

Grillner S, Markram H, De Schutter E, Silberberg G, LeBeau FE (2005) Microcircuits in action—from CPGs to neocortex. Trends Neurosci 28:525–533.

Halfon MS, Gisselbrecht S, Lu J, Estrada B, Keshishian H, Michelson AM (2002) New fluorescent protein reporters for use with the *Drosophila* Gal4 expression system and for vital detection of balancer chromosomes. Genesis 34:135–138.

Hoyle G (1985) Generation of motor activity and control of behaviour: the role of the neuromodulator octopamine and the orchestration hypothesis, In: Comparative insect physiology, biochemistry and pharmacology, Vol 5 (Kerkut GA, Gilbert L, eds), pp 607–621. Toronto: Pergamon.

Kiehn O, Kullander K (2004) Central pattern generators deciphered by molecular genetics. Neuron 41:317–321.

Kittel RJ, Wichman C, Rasse TM, Fouquet W, Schmidt M, Schmid A, Wagh DA, Pawlu C, Kellner RR, Willig KI, Hell SW, Buchner E, Heckmann M, Sigrist SJ (2006) Bruchpilot promotes active zone assembly, calcium channel clustering, and vesicle release. Science 312:1051–1054.

Kobayashi EA (2001) Role of catecholamine signaling in brain and nervous system functions: new insights from mouse molecular genetic study. J Investig Dermatol Symp Proc 6:115–121.

Kravitz EA, Huber R (2003) Aggression in invertebrates. Curr Opin Neurobiol 13:736–743.

Kutsukake M, Komatsu A, Yamamoto D, Ishiwa-Chigusa S (2000) A tyramine receptor gene mutation causes a defective olfactory behavior in *Drosophila melanogaster*. Gene 245:31–42.

Leitch B, Judge S, Pitman RM (2003) Octopaminergic modulation of synaptic transmission between an identified sensory afferent and flight motoneuron in the locust. J Comp Neurol 462:55–70.

Marder E, Bucher D (2001) Central pattern generators and the control of rhythmic movements. Curr Biol 11:R986–R996.

Marder E, Bucher D, Schulz DJ, Taylor AL (2005) Invertebrate central pattern generation moves along. Curr Biol 6:R685–R699.

Matheson T (1997) Octopamine modulates the responses and presynaptic inhibition of proprioceptive sensory neurones in the locust *Schistocerca gregaria*. J Exp Biol 200:1317–1325.

McClung C, Hirsh J (1999) The trace amine tyramine is essential for sensitization to cocaine in *Drosophila*. Curr Biol 9:853–860.

Mentel T, Duch C, Stypa H, Wegener G, Mueller U, Pflueger HJ (2003) Central modulatory neurons control fuel selection in flight muscle of migratory locust. J Neurosci 23:1109–1113.

Monastirioti M, Linn Jr CE, White K (1996) Characterization of *Drosophila* tyramine β-hydroxylase gene and isolation of mutant flies lacking octopamine. J Neurosci 16:3900–3911.

Nagaya Y, Kutsukake M, Chigusa SI, Komatsu A (2002) A trace amine, tyramine, functions as a neuromodulator in *Drosophila melanogaster*. Neurosci Lett 329:324–328.

Nusbaum MP, Blitz DM, Swensen AM, Wood D, Marder E (2001) The roles of co-transmission in neural network modulation. Trends Neurosci 24:146–154.

Orchard I, Ramirez JM, Lange AB (1993) A multifunctional role for octopamine in locust flight. Annu Rev Entomol 38:227–249.

Popova NK (2006) From genes to aggressive behavior: the role of the serotonergic system. BioEssays 28:495–503.

Ramirez JM, Orchard I (1990) Octopaminergic modulation of the forewing stretch receptor in the locust, *Locusta migratoria*. J Exp Biol 149:255–279.

Ramirez JM, Pearson KG (1991) Octopaminergic modulation of plateau potentials in the flight system of the locust. Brain Res 549:332–337.

Riemensperger T, Voller T, Stock P, Buchner E, Fiala A (2005) Punishment prediction by dopaminergic neurons in *Drosophila*. Curr Biol 16:1741–1747.

Roeder T (1999) Octopamine in invertebrates. Prog Neurobiol 59:533–561.

Saraswati S, Fox LE, Soll DR, Wu CF (2004) Tyramine and octopamine have opposite effects on the locomotion of *Drosophila* larvae. J Neurobiol 58:425–441.

Saudou F, Amlaiky N, Plassat JL, Borelli E, Hen R (1990) Cloning and characterization of *Drosophila* tyramine receptor. EMBO J 9:3611–3617.

Scheiner R, Plückhahn S, Oney B, Blenau W, Erber J (2002) Behavioral pharmacology of octopamine, tyramine, and dopamine in honey bees. Behav Brain Res 136:545–553.

Scholtissen B, Verhey FR, Steinbusch HW, Leentjens AF (2006) Serotonergic mechanisms in Parkinson's disease: opposing results from preclinical and clinical data. J Neural Transm 113:59–73.

Schwaerzel M, Monastirioti M, Scholz H, Friggi-Grelin F, Birman S, Heisenberg M (2003) Dopamine and octopamine differentiate between aversive and appetitive olfactory memories in *Drosophila*. J Neurosci 23:10495–10502.

Sombati S, Hoyle G (1984) Generation of specific behaviors in a locust by local release into neuropil of the natural neuromodulator octopamine. J Neurobiol 15:481–506.

Stevenson PA, Kutsch W (1987) A reconsideration of the central pattern generator concept for locust flight. J Comp Physiol A Neuroethol Sens Neural Behav Physiol 161:115–129.

Stevenson PA, Spoerhase-Eichmann U (1995) Localization of octopaminergic neurons in insects. Comp Biochem Physiol A Physiol 110:203–215.

Stevenson PA, Dyakonova V Rillich J, Schildberger K (2005) Octopamine

and experience-dependent modulation of aggression in crickets. J Neurosci 25:1431–1441.

Taylor C, Fricker AD, Devi LA, Gomes I (2005) Mechanisms of action of antidepressants: from neurotransmitter systems to signaling pathways. Cell Signal 17:549–557.

Wagh DA, Rasse TM, Asan E, Hofbauer A, Schwenkert I, Dürrbeck H, Buchner S, Dabauvalle MC, Schmidt M, Qin G, Wichmann C, Kittel R, Sigrist SJ, Buchner E (2006) Bruchpilot, a protein with homology to ELKS/CAST, is required for structural integrity and function of synaptic active zones in *Drosophila*. Neuron 49:833–844.

Wegener G (1996) Flying insects: model systems in exercise physiology. Experientia 52:404–412.

White K, Grether ME, Abrams JM, Young L, Farrell K, Steller H (1994) Genetic control of programmed cell death in *Drosophila*. Science 264:677–683.

Wilson DM (1961) The central nervous control of locust flight. J Exp Biol 38:471–490.

Wilson DM (1966) Central nervous mechanisms for the generation of rhythmic behavior in arthropods. Symp Soc Exp Biol 20:199–228.

Wilson DM, Wyman RJ (1965) Motor output patterns during random and rhythmic stimulation of locust thoracic ganglia. Biophys J 5:121–143.

Zhou L, Schnitzler A, Agapite J, Schwartz LM, Steller H, Nambu JR (1997) Cooperative functions of the reaper and head involution defective genes in the programmed cell death of *Drosophila* central nervous system midline cells. Proc Natl Acad Sci USA 94:5131–5136.

Video Article

Operant learning of *Drosophila* at the torque meter

Björn Brembs
Institute of Biology - Neurobiology, Freie Universität Berlin

Correspondence to: Björn Brembs at bjoern@brembs.net

URL: http://www.jove.com/index/Details.stp?ID=731
DOI: 10.3791/731
Citation: Brembs B. (2008). Operant learning of Drosophila at the torque meter. JoVE. 16. http://www.jove.com/index/Details.stp?ID=731, doi: 10.3791/731

Abstract

For experiments at the torque meter, flies are kept on standard fly medium at 25 C and 60% humidity with a 12hr light/12hr dark regime. A standardized breeding regime assures proper larval density and age-matched cohorts. Cold-anesthetized flies are glued with head and thorax to a triangle-shaped hook the day before the experiment. Attached to the torque meter via a clamp, the fly's intended flight maneuvers are measured as the angular momentum around its vertical body axis. The fly is placed in the center of a cylindrical panorama to accomplish stationary flight. An analog to digital converter card feeds the yaw torque signal into a computer which stores the trace for later analysis. The computer also controls a variety of stimuli which can be brought under the fly's control by closing the feedback loop between these stimuli and the yaw torque trace. Punishment is achieved by applying heat from an adjustable infrared laser.

Protocol

Fly medium

The composition of the fly food is critical for learning (Guo et al., 1996):

- Water 1000 ml
- Cornmeal 180 g
- Soybean 10 g
- Yeast 18.5 g
- Agar 7.5 g
- Molasses 40 g
- Syrup (sugar beet) 40 g
- Nipagin 2.5 g

Every vial is supplied with a dab of fresh, living yeast paste and a piece of filter paper to provide an additional surface for flies and pupae.

Fly breeding and staging

The following procedure is performed every day, leading to precisely staged animals grown at the appropriate density. All newly ecclosed flies since the last procedure on the previous day are collected for breeding and experiments. The oldest vials without any remaining living pupae are discarded. Four day old flies are added to a fresh vial for egg deposition over night. The density of female flies should be approximately 20 for each vial, adjusted for the size of the vial and the strain used. The ideal density is one that is high enough for the fly medium to liquefy during the larval stages and low enough such that all larvae have pupated before the first flies ecclose. The egg-laying flies from the previous day are removed and discarded.

Fly preparation

Flies are kept on standard cornmeal/molasses medium as described above at 25°C and 60% humidity with a 12hr light/12hr dark regime. After briefly immobilizing 24-48h old flies by cold-anaesthesia, the flies are glued (SuperGlue UV glass adhesive, 505127A, Pacer Technology, Cucamonga, Ca., USA) with head and thorax to a triangle-shaped copper hook (diameter 0.05mm) the day before the experiment. The animals are then kept individually overnight in small moist chambers containing a few grains of sucrose.

Apparatus

The core device of the set-up is the torque compensator (torque meter) (Götz, 1964). It measures a fly's angular momentum around its vertical body axis, caused by intended flight manoeuvres. The fly, glued to the hook as described above, is attached to the torque meter via a clamp to accomplish stationary flight in the centre of a cylindrical panorama (arena, diameter 58mm), which is homogeneously illuminated from behind. The light source is a 100W, 12V tungsten-iodine bulb. For green and blue illumination of the arena, the light is passed through monochromatic broad band Kodak Wratten gelatin filters (#47 and #99, respectively). Filters can be exchanged by a fast solenoid within 0.1s. Alternatively, the arena is illuminated with 'daylight' by passing it through a blue-green filter (Rosco "surfblue" No. 5433), or no filter at all. The transmission spectrum of the Rosco blue-green filter used in this study is equivalent to that of a BG18 filter (Schott, Mainz) and constitutes an intermediate between the Kodak blue and green filters (Brembs and Hempel de Ibarra, 2006; Liu et al., 1999). The arena can be rotated around the fly using a computer controlled electric motor. In such a 'flight-simulator' situation, the angular velocity of the arena is proportional to, but directed against the fly's yaw torque (coupling factor K=-11°/s•10^{-10}Nm). This enables the fly to stabilize the panorama and to control its angular orientation. This virtual 'flight direction' (i.e., arena position) is recorded continuously via a circular potentiometer (Novotechnik, A4102a306). An analogue to digital converter card (PCL812; Advantech Co.) feeds the arena position and the yaw torque signal into a computer which stores the traces (sampling

frequency 20Hz) for later analysis. Punishment is achieved by applying heat from an adjustable infrared laser (825 nm, 150 mW), directed from behind and above onto the fly's head and thorax. The laser beam is pulsed (approx. 200ms pulse width at ~4Hz) and its intensity reduced to assure the survival of the fly.

Experiments

Pattern learning

For the traditional pattern-learning experiment (Dill and Heisenberg, 1995; Dill et al., 1993, 1995; Liu et al., 2006; Liu et al., 1998; Liu et al., 1999; Wolf and Heisenberg, 1991), four black, T-shaped patterns of alternating orientation (i.e. two upright and two inverted) are evenly spaced on the arena wall (pattern width ψ=40°, height θ=40°, width of bars=14°, as seen from the position of the fly). A computer program divides the 360° of the arena into 4 virtual 90° quadrants, the centers of which are denoted by the patterns. The flies control the angular position of the patterns with its yaw torque (flight simulator situation). During training, heat punishment is made contiguous with the appearance of one of the pattern orientations in the frontal visual field. Reinforcement of each pattern is always equalized within groups. During test, the heat is permanently switched off and the fly's pattern preference recorded.

Color learning

Color learning is performed as described before (Brembs and Heisenberg, 2000; Brembs and Hempel de Ibarra, 2006; Brembs and Wiener, 2006; Wolf and Heisenberg, 1997). The arena is divided into four virtual 90° quadrants, the centers of which are denoted by four identical vertical stripes (width ψ=14°, height θ=40°). The fly is controlling the angular position of the four identical stripes with its yaw torque as described for the T-shaped patterns above. The color of the illumination of the whole arena is changed whenever one of the virtual quadrant borders passes a point in front of the fly. During training, heat punishment is made contingent on one of the two colors. During test, the heat is permanently switched off and he fly's color preference recorded. Of course, the colors can be combined with patterns for compound conditioning (Brembs and Heisenberg, 2001).

Yaw torque learning

Yaw torque learning is performed as previously described (Brembs and Heisenberg, 2000; Heisenberg and Wolf, 1993). The fly's spontaneous yaw torque range is divided into a ‘left' and ‘right‘ domain, approximately corresponding to either left or right turns. There are no patterns on the arena wall. During training, heat is applied whenever the fly's yaw torque is in one domain and switched off when the torque passes into the other. In the test phases, heat is permanently switched off and the fly's choice of yaw torque domains is recorded.

Composite learning

Composite learning is an extension of yaw torque learning, as described before (Brembs and Heisenberg, 2000). Basically, yaw torque learning and color learning are combined in an experiment with equivalent operant (yaw torque) and classical (colors) predictors. During training, the fly is heated whenever the fly's yaw torque passes into the domain associated with punishment. Whenever the fly switches yaw torque domains, not only temperature but also arena coloration is changed (from green to blue or vice versa). Thus, yaw torque domain and color serve as equivalent predictors of heat. In the test phases, heat is permanently switched off and only the fly's choice of yaw torque domains/colors is recorded.

Discussion

This experimental setup combines superb control over experimental circumstances with an advanced genetic model organism. Using the procedures described in this presentation, the molecular and neurobiological underpinnings of a variety of behavioral traits can be investigated, including, but not limited to, the mechanisms of spontaneous behavior generation, operant and classical conditioning, pattern recognition, color vision or course control.

Discussion

This experimental setup combines superb control over experimental circumstances with an advanced genetic model organism. Using the procedures described in this presentation, the molecular and neurobiological underpinnings of a variety of behavioral traits can be investigated, including, but not limited to, the mechanisms of spontaneous behavior generation, operant and classical conditioning, pattern recognition, color vision or course control.

Acknowledgements

The original design of the torque compensator originates with Karl Götz. The particular setup in this presentation is to a large extent on loan and was originally developed by Martin Heisenberg and Reinhard Wolf. I am especially indebted to these two persons for their continued support, encouragement and expertise.

References

1. Guo, A. et al. Conditioned visual flight orientation in Drosophila; Dependence on age, practice and diet. Learn. Mem. 3, 49-59 (1996).

2. Götz, K. G. Optomotorische Untersuchung des visuellen Systems einiger Augenmutanten der Fruchtfliege Drosophila. Kybernetik. 2, 77-92 (1964).

3. Liu, L., Wolf, R., Ernst, R., & Heisenberg, M. Context generalization in Drosophila visual learning requires the mushroom bodies. Nature 400, 753-756 (1999).

4. Brembs, B. & Hempel de Ibarra, N. Different parameters support generalization and discrimination learning in Drosophila at the flight simulator. Learn. Mem. 13, 629-637 (2006).

5. Liu, G. et al. Distinct memory traces for two visual features in the Drosophila brain. Nature 439, 551-556 (2006).

6. Wolf, R. & Heisenberg, M. Basic organization of operant behavior as revealed in Drosophila flight orientation. J. Comp. Physiol. A Neuroethol. Sens. Neural. Behav. Physiol. 169, 699-705 (1991).

7. Liu, L. et al. Conditioned visual flight orientation in Drosophila melanogaster abolished by benzaldehyde. Pharmacol Biochem Behav 61, 349-355 (1998).

8. Dill, M. & Heisenberg, M. Visual pattern memory without shape recognition. Philos. Trans. R. Soc. Lond. B. Biol. Sci. 349, 143-152 (1995).

9. Dill, M., Wolf, R., & Heisenberg, M. Visual pattern recognition in Drosophila involves retinotopic matching. Nature. 365, 751-753 (1993).

10. Dill, M., Wolf, R., & Heisenberg, M. Behavioral analysis of Drosophila landmark learning in the flight simulator. Learn. Mem. 2, 152-160 (1995).

11. Wolf, R. & Heisenberg, M. Visual Space from Visual Motion: Turn Integration in Tethered Flying Drosophila. Learn. Mem. 4, 318-327 (1997).

12. Brembs, B. & Heisenberg, M. The operant and the classical in conditioned orientation in Drosophila melanogaster at the flight simulator. Learn. Mem. 7, 104-115 (2000).

13. Brembs, B. & Wiener, J. Context generalization and occasion setting in Drosophila visual learning. Learn. Mem. 13, 618-628 (2006).

14. Brembs, B. & Heisenberg, M. Conditioning with compound stimuli in Drosophila melanogaster in the flight simulator. J Exp Biol 204, 2849-2859 (2001).

15. Heisenberg, M. & Wolf, R. The sensory-motor link in motion-dependent flight control of flies. Rev. Oculomot. Res. 5, 265-283 (1993).

Current Biology *18*, 1168–1171, August 5, 2008 DOI 10.1016/j.cub.2008.07.041

Report

Double Dissociation of PKC and AC Manipulations on Operant and Classical Learning in *Drosophila*

Björn Brembs[1,*] and Wolfgang Plendl[2,3]
[1]Freie Universität Berlin
Institut für Biologie – Neurobiologie
Königin-Luise Str. 28/30
14195 Berlin
Germany
[2]Lehrstuhl für Genetik und Neurobiologie, Biozentrum
Am Hubland
Universität Würzburg
97074 Würzburg
Germany

Summary

Learning about relationships between stimuli (i.e., classical conditioning [1]) and learning about consequences of one's own behavior (i.e., operant conditioning [2]) constitute the major part of our predictive understanding of the world. Since these forms of learning were recognized as two separate types 80 years ago [3], a recurrent concern has been the issue of whether one biological process can account for both of them [4–9]. Today, we know the anatomical structures required for successful learning in several different paradigms, e.g., operant and classical processes can be localized to different brain regions in rodents [9] and an identified neuron in *Aplysia* shows opposite biophysical changes after operant and classical training, respectively [5]. We also know to some detail the molecular mechanisms underlying some forms of learning and memory consolidation. However, it is not known whether operant and classical learning can be distinguished at the molecular level. Therefore, we investigated whether genetic manipulations could differentiate between operant and classical learning in *Drosophila*. We found a double dissociation of protein kinase C and adenylyl cyclase on operant and classical learning. Moreover, the two learning systems interacted hierarchically such that classical predictors were learned preferentially over operant predictors.

Results

We subjected *rut*^{2080} mutants (affecting a type I adenylyl cyclase [AC] that is regulated by Ca^{2+}/Calmodulin and G protein) and transgenic flies expressing an inhibitory pseudosubstrate of protein kinase C (PKCi) under the control of a heat-shock promoter to three experimental procedures: one with only a classical predictor, one with only an operant predictor, and one with both predictors. The Rutabaga type I AC is one of the first learning genes identified and required for various forms of classical learning [10, 11]. It is unknown whether "pure" operant learning (without any classical predictors [4]) also depends on this AC. Flies expressing PKCi have deficits in modifying their behavior after negative feedback but show intact memory of the stimulus predicting the feedback [12]. Therefore, PKC was considered a likely candidate gene involved in operant learning. In all three experiments, *Drosophila* fruit flies were tethered and suspended at a torque meter measuring the attempts of the flies to turn left or right (yaw torque). An infrared light beam served as an aversive stimulus to train the flies to discriminate between a punished and an unpunished situation. Each fly was trained on one of three different discriminations: (1) only with color as a classical predictor (blue or green; Figures 1A and 1B), (2) only with yaw torque as an operant predictor (left- or right-turning; Figures 2A and 2B), or (3) with a composite of both predictors (Figures 3A and 3B). For details on the experimental procedures, see Supplemental Data (available online) and [13]. Importantly, in all experiments, heat avoidance was normal in all strains (data not shown).

First, we tested the flies for learning the classical color predictor alone (Figure 1C). As expected, *rut* flies were deficient in the paradigm with only a classical predictor ($t_{15} = -0.5$, $p < 0.7$). Wild-type control flies showed normal classical learning ($t_{25} = 2.8$, $p < 0.01$), as did the transgenic flies expressing PKCi ($t_{19} = 2.6$, $p < 0.02$) and the uninduced control flies ($t_{22} = 2.4$, $p < 0.03$). The results were reversed in the strictly operant paradigm (Figure 2C). Despite failing all associative and many nonassociative learning tasks until now, *rut* flies show unimpaired operant behavioral learning ($t_{16} = 4.3$, $p < 0.001$). If anything, learning is slightly enhanced over wild-type control flies ($t_{29} = 3.0$, $p < 0.006$; see also Supplemental Data). In contrast, PKCi-induced flies do not show any behavioral learning ($t_{22} = 0.2$, $p < 0.9$). This deficit is specifically caused by the expression of PKCi because uninduced flies do not show this impairment ($t_{19} = 8.4$, $p < 0.001$) and neither do the heterozygous parental control strains (het. cont. HS: $t_{42} = 4.6$, $p < 0.001$; het. cont. noHS: $t_{40} = 5.7$, $p < 0.001$). With PKC and AC being differentially involved in operant and classical learning, respectively, the final experiment was performed to reveal their relative contributions in an ethologically more relevant, composite learning situation containing both operant and classical predictors (Figure 3C). The failure only of *rut* flies ($t_{16} = 0.7$, $p < 0.5$) and not of PKCi-induced ($t_{26} = 2.1$, $p < 0.05$) or control flies (wild-type: $t_{31} = 5.1$, $p < 0.001$; PKCi noHS: $t_{20} = 3.6$, $p < 0.002$) to master the composite task is evidence that in such learning situations, the classical predictor is learned preferentially over the operant predictor.

Discussion

We found a double dissociation of AC and PKC manipulations on classical and operant learning. Flies devoid of *rut*-AC, despite failing all associative learning tasks until now, perform well in operant learning without predictive stimuli, even outperforming wild-type flies (Figure S1). Conversely, manipulating PKC during training affects operant, but not classical, learning. This is consistent with previous reports showing that PKC manipulations have no effect during training but do have an effect in the maintenance of memory after classical training [14]. Our data clarify and extend another observation [12] in that

*Correspondence: bjoern@brembs.net
[3]Present address: Max-Planck-Institut für Psychiatrie, München, Germany

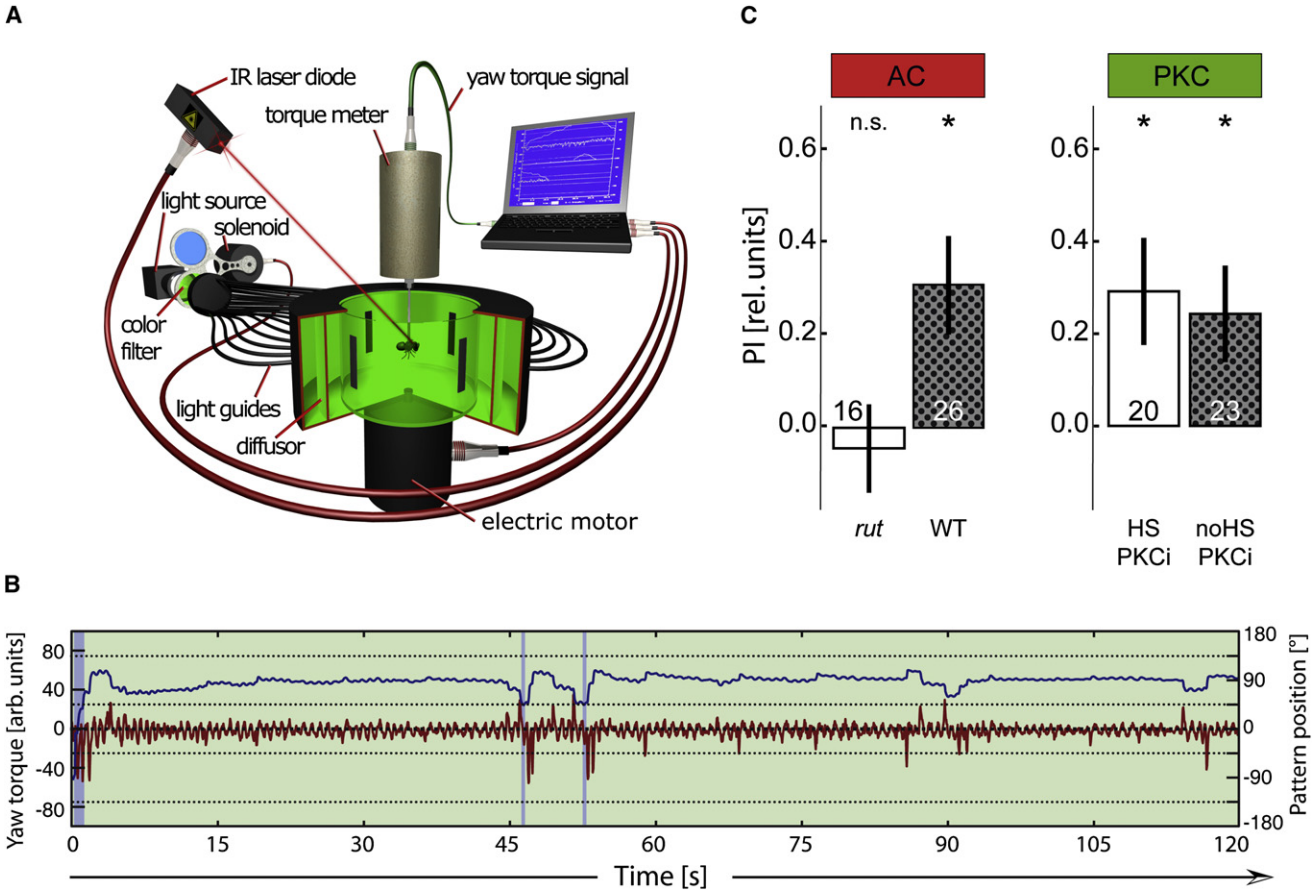


Figure 1. Manipulation of AC, but Not of PKC, Disrupts Learning of a Classical Predictor

(A) Experimental setup. The fly controls the angular position of a drum with four identical vertical bars in a flight-simulator-like situation. The coloration of the arena is switched between bars, such that flying toward one pair of opposing bars leads to green coloration and toward the other pair to blue coloration. During training, heat is made contingent on one color, irrespective of the turning maneuver that changed flight direction.

(B) Sample data from a wild-type fly during the first test period after the final training with heat on blue coloration. The fly uses both left- and right-turning maneuvers (red trace) to change flight direction (blue trace) and hence coloration of the environment (background color of the graph). The fly shows a clear preference for green with only brief excursions into flight directions that lead to blue color, even though the heat is switched off.

(C) Pooled performance indices (PI) from the first test period after training. In this and all subsequent bar graphs: Means are displayed, and error bars represent the standard error of the mean (SEM). Numbers at bars indicate the number of animals. The following abbreviations are used: *rut*, *rut* mutant flies affecting AC; WT, wild-type; HS PKCi, heat-shock-induced expression of the specific PKC inhibitor; noHS PKCi, PKCi expression not induced; and n.s., not significant. *, $p < 0.05$.

expressing PKCi selectively affects the capacity for storing behavioral modifications (operant learning) but leaving both classical learning and the capacity to control external stimuli by ongoing behavioral modifications (operant behavior) intact. Recent evidence from *Aplysia* also implicates PKC in operant learning, suggesting that this is a conserved function of PKC [15]. The discovery of PKC underlying operant learning opens the experimental possibility of localizing the structures where PKC is necessary and sufficient for operant learning in the fly brain, a strategy that was used to map engrams in visual and olfactory learning [10, 11]. Our experiments do not provide any evidence for crosstalk between the AC and PKC pathway, leaving the possibility that operant and classical learning may be based on two largely separate molecular processes, which could occur in the same neuron [5]. The hierarchical interaction between operant and classical components in composite learning situations contrasts with the symmetry in which two equivalent classical predictors are learned in compound conditioning [16]. This hierarchy of multiple memory systems also suggests how the separate molecular basis for operant learning could be missed despite many years of research: Procedurally operant paradigms are dominated by the formation of a biologically classical memory if environmental predictors are available [4]. For instance, our results predict the deficit of *rut* mutant flies in another procedurally operant paradigm designed to screen for operant mutants (the heatbox [7]), because of the analogy of the spatial cues in the heatbox with the color cues used here. In other words, as soon as predictive stimuli are present in operant-learning situations, not only do these stimuli become equivalent to classical stimuli with respect to their independence from the behavior with which they were learned [4], but these composite experiments also cannot be distinguished genetically from classical experiments any more.

Our data and the current literature are consistent with the hypothesis that operant and classical learning can be distinguished by the differential spatiotemporal requirement of several AC and PKC isoform activities, respectively.

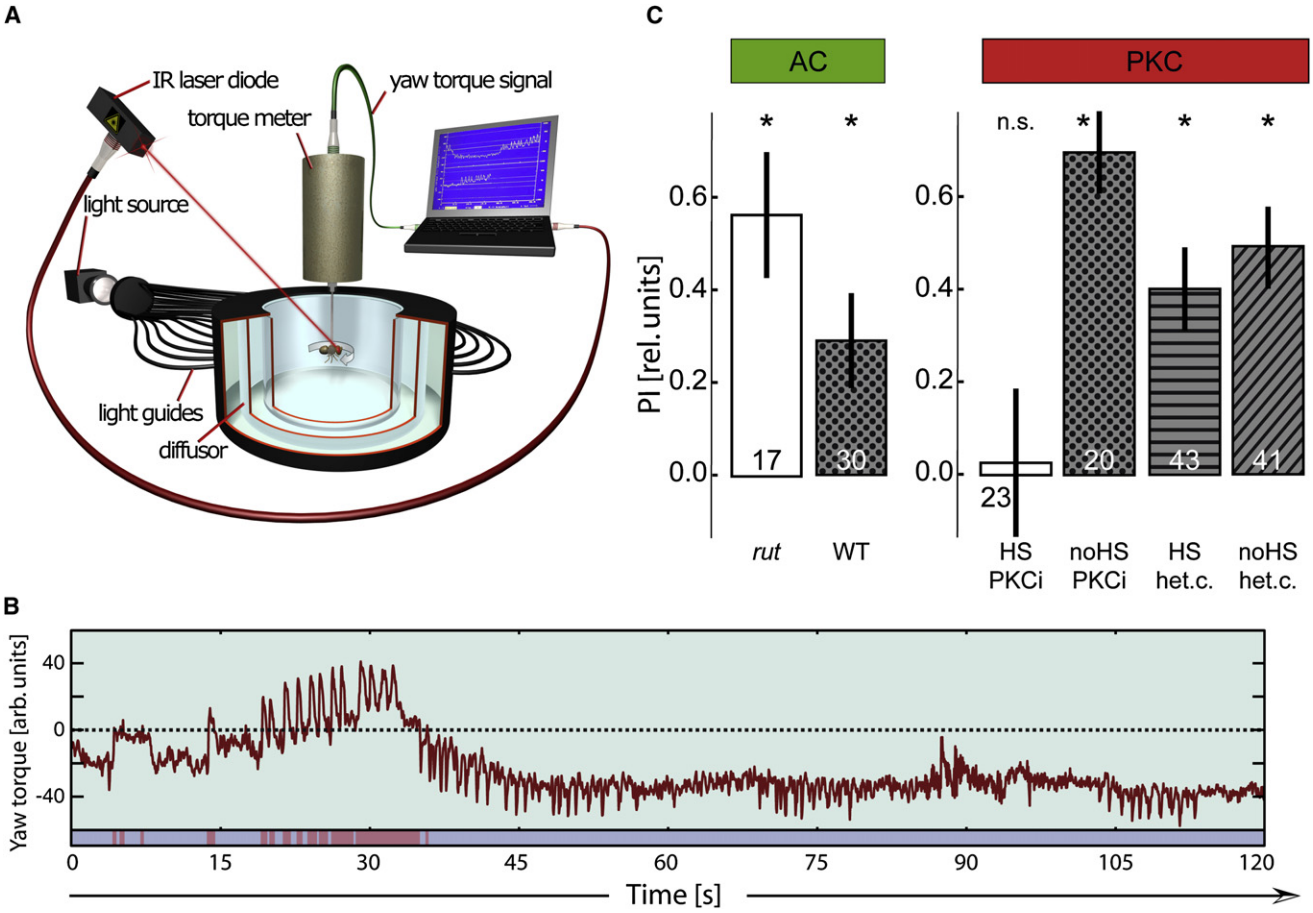


Figure 2. Manipulation of PKC, but Not of AC, Disrupts Learning of an Operant Predictor

(A) Experimental setup. There are no visual cues for the fly. During training, heat is made contingent on either left- or right-turning yaw torque.
(B) Sample data from a wild-type fly during the first test period after the final training with heat on positive (right-turning) yaw torque. The fly only briefly generates right-turning yaw torque during the test phase (unsaturated red/blue bar underneath dark red yaw-torque trace), even though the heat is switched off.
(C) Pooled performance indices (PI) from the first test period after training. The following abbreviations are used: HS het.c., heat-shock-treated heterozygous parental controls strain; noHS het.c., heterozygous parental control strain without heat shock.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and one figure and can be found with this article online at http://www.current-biology.com/cgi/content/full/18/15/1168/DC1/.

Acknowledgments

We thank Martin Heisenberg and Henrike Scholz for providing fly strains and Tilman Franke for creating the 3D renderings of the experimental setup. The feedback from four anonymous referees improved the quality of the manuscript tremendously. Author contributions: B.B. designed and performed the experiments, analyzed the data and wrote the manuscript; W.P. first examined *rut* and PKCi strains in pilot experiments and has read the final version of the manuscript.

Received: April 30, 2008
Revised: July 8, 2008
Accepted: July 8, 2008
Published online: July 31, 2008

References

1. Pavlov, I.P. (1927). Conditioned Reflexes (Oxford: Oxford University Press).
2. Skinner, B.F. (1938). The Behavior of Organisms (New York: Appleton).
3. Miller, S., and Konorski, J. (1928). Sur une forme particuliere des reflexes conditionnels. C. r. Soc. Biol. *99*, 1155–1157.
4. Brembs, B., and Heisenberg, M. (2000). The operant and the classical in conditioned orientation in *Drosophila melanogaster* at the flight simulator. Learn. Mem. *7*, 104–115.
5. Lorenzetti, F.D., Mozzachiodi, R., Baxter, D.A., and Byrne, J.H. (2006). Classical and operant conditioning differentially modify the intrinsic properties of an identified neuron. Nat. Neurosci. *9*, 17–29.
6. Gormezano, I., and Tait, R.W. (1976). The Pavlovian analysis of instrumental conditioning. Pavlov. J. Biol. Sci. *11*, 37–55.
7. Diegelmann, S., Zars, M., and Zars, T. (2006). Genetic dissociation of acquisition and memory strength in the heat-box spatial learning paradigm in Drosophila. Learn. Mem. *13*, 72–83.
8. Brembs, B., Lorenzetti, F.D., Reyes, F.D., Baxter, D.A., and Byrne, J.H. (2002). Operant reward learning in Aplysia: neuronal correlates and mechanisms. Science *296*, 1706–1709.
9. Ostlund, S.B., and Balleine, B.W. (2007). Orbitofrontal cortex mediates outcome encoding in pavlovian but not instrumental conditioning. J. Neurosci. *27*, 4819–4825.
10. Liu, G., Seiler, H., Wen, A., Zars, T., Ito, K., Wolf, R., Heisenberg, M., and Liu, L. (2006). Distinct memory traces for two visual features in the *Drosophila* brain. Nature *439*, 551–556.
11. Zars, T., Fischer, M., Schulz, R., and Heisenberg, M. (2000). Localization of a short-term memory in Drosophila. Science *288*, 672–675.

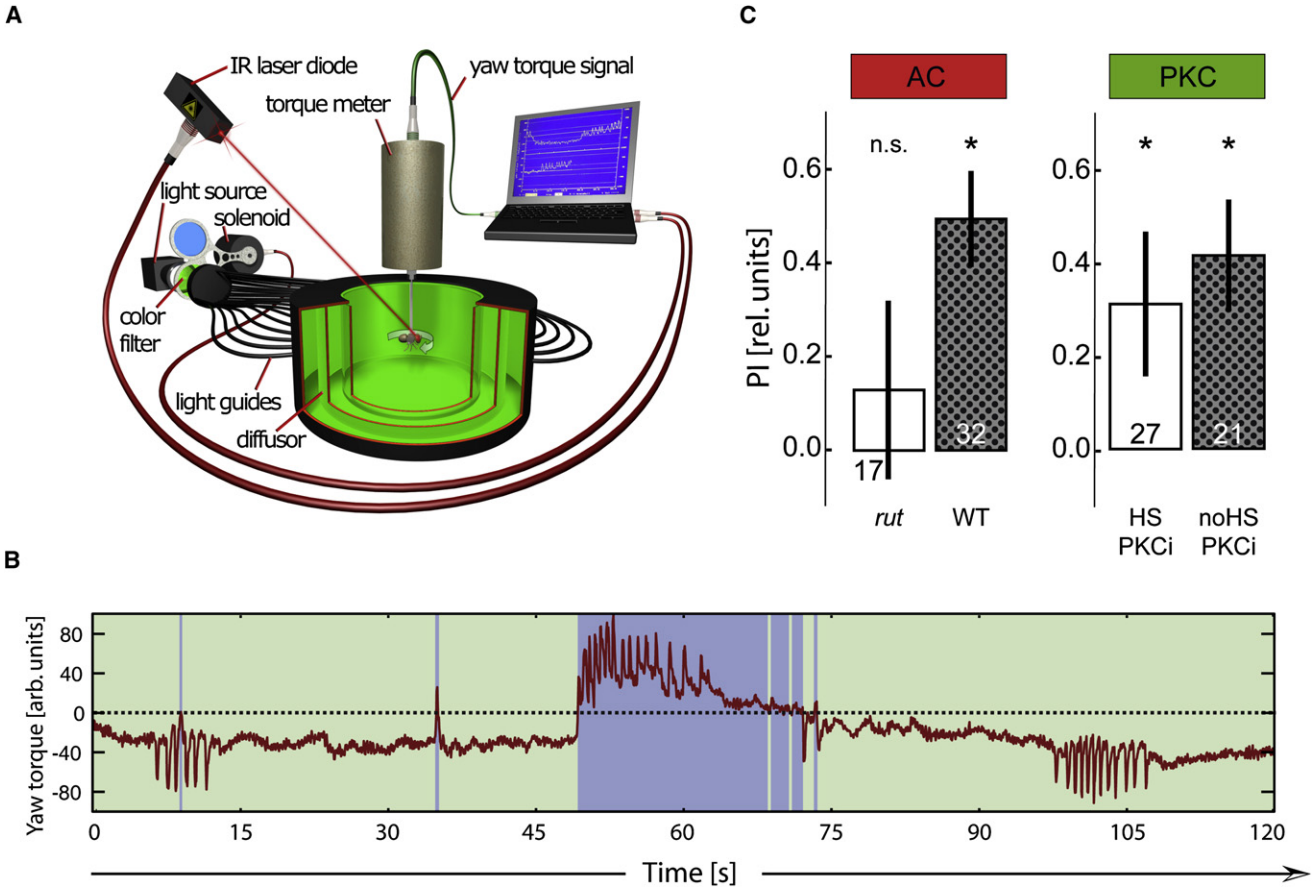


Figure 3. Learning a Classical Predictor Dominates over Learning an Operant Predictor

(A) Experimental design. Throughout the experiment, one yaw-torque domain is coupled to one color and the other to the other color (e.g., right turning causes green illumination and left turning blue illumination of the environment). During training, heat is made contingent on one of the two combinations of yaw torque and color.

(B) Sample data from a wild-type fly during the first test period after the final training with heat on positive (right-turning) yaw torque (red trace) and blue illumination (background coloration). The fly shows the preference for yaw-torque domain or color and only briefly ventures into the previously punished situation, even though the heat is switched off.

(C) Pooled performance indices (PI) from the first test period after training.

12. Kane, N.S., Robichon, A., Dickinson, J.A., and Greenspan, R.J. (1997). Learning without performance in PKC-deficient Drosophila. Neuron *18*, 307–314.
13. Brembs, B. (2008). Operant learning of Drosophila at the torque meter. J. Vis. Exp. *16*, 710.3791/3731.
14. Drier, E.A., Tello, M.K., Cowan, M., Wu, P., Blace, N., Sacktor, T.C., and Yin, J.C.P. (2002). Memory enhancement and formation by atypical PKM activity in *Drosophila melanogaster*. Nat. Neurosci. *5*, 316–324.
15. Lorenzetti, F., Baxter, D., and Byrne, J. (2008). Molecular mechanisms underlying a cellular analogue of operant reward learning. Neuron *59*, in press.
16. Brembs, B., and Heisenberg, M. (2001). Conditioning with compound stimuli in *Drosophila melanogaster* in the flight simulator. J. Exp. Biol. *204*, 2849–2859.

Mushroom-bodies mediate hierarchical interactions between operant and classical learning systems in *Drosophila* visual learning

Björn Brembs
Freie Universität Berlin, Institut für Biologie – Neurobiologie, Königin Luise Str. 28-30, 14195 Berlin
Email: bjoern@brembs.net
Tel: +49 30 838 55050, fax: +49 30 838 55455

Number of pages: 15
Number of figures: 4
Number of words: 4,434

Key words: invertebrate, fruit fly, insect, instrumental, Pavlovian, conditioning
Running title: habit formation and generalization in *Drosophila*

Acknowledgements
I am grateful to Martin Heisenberg, Reinhard Wolf and Randolf Menzel for critical discussions and comments on earlier versions of the manuscript. Fly strains were generously provided by Martin Heisenberg, Hiromu Tanimoto and Scott Waddell. Tilman Franke created the 3D renderings of the experimental setup using PovRay. I am especially indebted to Jochen Pflüger for supporting me financially, with labspace, advice and encouragement in times of great need.

Abstract

Previous studies on fruit fly learning have found a hierarchical interaction between operant and classical components in learning situations where both operant and classical predictors are present (Brembs and Plendl, 2008). In this situation, the classical component dominated the learning task. In the current study, experiments using transgenic flies with impaired mushroom-body function revealed that the mushroom-body α and β lobes mediate this hierarchical interaction by inhibiting operant learning when a classical stimulus is present. This inhibition enables generalization of the classical memory and prevents premature habit formation. Extended training in wildtype flies produced a phenocopy of mushroom-body impaired flies, such that generalization was abolished and goal-directed actions were transformed into habitual responses (habit formation). The current data are consistent with the hypothesis that operant learning situations consisting of both operant and classical predictors lead to a hierarchical and reciprocal interaction between two memory systems in the fly's brain. The classical system inhibits memory storage by the operant system via the mushroom-bodies. The operant system, in turn, facilitates memory storage by the classical system via unknown, non-mushroom-body pathways. Such an organization can help explain how the presence of predictive stimuli in a procedurally operant learning task can transform the task into one that is genetically indistinguishable from a classical learning task.

Introduction

The relationship between operant and classical conditioning has been a recurrent concern, first in psychology and later also in neuroscience. The 80 year-old debate concerned both the question of whether one biological process could account for both phenomena and, if not, how the two processes would interact (Miller and Konorski, 1928; Rescorla and Solomon, 1967; Gormezano and Tait, 1976; Heisenberg et al., 2001; Brembs et al., 2002; Brembs et al., 2004). Recently, several publications have reported a separation of operant and classical learning not only at the behavioral, but also at the anatomical, cellular and genetic level, settling that part of the debate (Lorenzetti et al., 2006; Ostlund and Balleine, 2007; Brembs and Plendl, 2008; Lorenzetti et al., 2008). One of these studies also addressed the second aspect of the debate by discovering a hierarchical interaction between operant and classical processes in learning situations with both operant and classical components (Brembs and Plendl, 2008). The interaction appeared to be such that with operant behavior and classical stimuli predicting the reinforcer equivalently, the animals preferentially learned the classical stimuli. This relationship was discovered by testing *rutabaga* (*rut*) mutant flies in such composite situations. The Rutabaga protein, a type I adenylyl cyclase (AC) that is regulated by Ca^{2+}/Calmodulin and G protein, is required for learning a classical stimulus but not for operant learning. In composite learning situations with equivalent operant and classical predictors, *rut* mutant flies are impaired as well, whereas flies expressing a specific inhibitor of protein kinase C (PKCi) are not. Flies expressing PKCi, on the other hand, are defective in learning operant but not classical predictors (Brembs and Plendl, 2008). In this study, I investigated the biological nature of this hierarchy.

Materials and Methods

Fly strains: Wild-type strain *Berlin* (WT) and *rutabaga* mutant strain rut^{2080} were used for this study. Sweeney et al. (1995) developed a method that constitutively blocks synaptic transmission by expressing the catalytic subunit of bacterial tetanus toxin (TNT) in target neurons in the *Drosophila* brain using the P[GAL4] technique (Brand and Perrimon, 1993). Because of the effects of MB function on context generalization (Liu et al., 1999; Brembs and Wiener, 2006), we used the TNT transgene to block synaptic output from the MB. Despite some technical issues which have been raised recently (Rister and Heisenberg, 2006; Thum et al., 2006), we favored TNT over the temperature-sensitive *shibire* effector, because of the heat punishment in our paradigm. We use the trans-heterozygote offspring from the driver (mb247) and the reporter strain (UAS_{GAL4}-TNT) for our studies as described previously

(Sweeney et al., 1995; Baier et al., 2002; Brembs and Wiener, 2006). The heterozygote offspring from crossing driver and reporter strain, respectively, to Canton-S wildtype flies served as genetic controls for these experiments. In addition to the mb247 line we also used the line 17D which only expresses in the α and β lobes, but not in the γ lobes (Martin et al., 1998). Any shared effects between the two crosses can therefore be attributed to the overlapping expression pattern in the MB between the two driver lines. To test for the specificity of the effects in the MB GAL4 lines, we tested a non-MB line, c205, which drives TNT expression in the F5 neurons of the fan-shaped body (Liu et al., 2006).

Fly preparation. Flies were kept on standard cornmeal/molasses medium (Guo et al., 1996) at 25°C and 60% humidity with a 12hr light/12hr dark regime. After briefly immobilizing 24-48h old female flies by cold-anesthesia, the flies were glued (Locktite UV glass glue) with head and thorax to a triangle-shaped copper hook (diameter 0.05mm) the day before the experiment (Brembs, 2008). The animals were then kept individually overnight in small moist chambers containing a few grains of sucrose.

Apparatus. The core device of the set-up is the torque compensator (torque meter) (Götz, 1964). It measures a fly's angular momentum around its vertical body axis, caused by intended flight maneuvers. The fly, glued to the hook as described above, is attached to the torque meter via a clamp to accomplish stationary flight in the center of a cylindrical panorama (arena, diameter 58mm), which is homogeneously illuminated from behind (Fig. 1). The light source is a 100W, 12V tungsten-iodine bulb. For green and blue illumination of the arena, the light is passed through monochromatic broad band Kodak Wratten gelatin filters (#47 and #99, respectively). Filters can be exchanged by a fast solenoid within 0.1s. Alternatively, the arena is illuminated throughout the experiment with ‘daylight’ by passing it through a blue-green filter (Rosco “surfblue” No. 5433). The transmission spectrum of the Rosco filter used in this study constitutes an intermediate between the Kodak blue and green filters (Brembs and Hempel de Ibarra, 2006). An analog to digital converter card (PCL812; Advantech Co.) feeds the yaw torque signal into a computer which stores the trace (sampling frequency 20Hz) for later analysis. Punishment is achieved by applying heat from an adjustable infrared laser (825 nm, 150 mW), directed from behind and above onto the fly's head and thorax (Brembs, 2008). The laser beam is pulsed (approx. 200ms pulse width at ~4Hz) and its intensity reduced to assure the survival of the fly.

Experimental design. Each fly was used only once. The time-course of the experiment was divided into consecutive periods of 2 minutes duration. Depending on whether heat may

be applied during such a period, it is termed a training period (heating possible) or a test period (heat off). Standard experiments consisted of two pre-test periods (labeled PI_1 and PI_2) 4 training periods (PI_3, PI_4, PI_6 and PI_7) and three memory test periods (PI_5, PI_8 and PI_9). Only in experiments testing the generalization of the classical memory, PI_8 was a 60s familiarization training and PI_9 was scored as memory test. For experiments with extended training, the experimental time course was essentially repeated such that in total four additional training periods (PI_9, PI_{10}, PI_{12}, PI_{13}) followed training-PI_7, as well as five test periods (PI_8, PI_{11}, PI_{14}, PI_{15}). Only in experiments testing the generalization of the classical memory, PI_{14} was a 60s familiarization training and PI_{15} was scored as memory test. Depicted are always the PI's of the first two minutes after the last training period. All animals were trained with operant and classical predictors as described before (Brembs and Heisenberg, 2000; Brembs and Plendl, 2008). In brief, the fly's spontaneous yaw torque range was divided into a 'left' and 'right' domain, approximately corresponding to either left or right turns (Wolf and Heisenberg, 1991). Heat punishment and arena coloration were made contingent on this behavior such that, e.g., left turning lead to green arena illumination and heat on, whereas right turning lead to blue arena illumination and heat off. Punishment of yaw torque domains/colors was always counterbalanced. For the standard duration experiments, this situation lasted until PI_7, the final training period. Only in experiments with extended training duration was this situation prolonged until PI_{13}. After the final training period, the animals were divided into three different groups. The three groups essentially follow the three experiments described before (Brembs and Plendl, 2008). Group 1 (control) was tested in the composite situation without heat. Group 2 was tested without heat or colors for spontaneous choice of yaw torque domains (operant component). Group 3 was tested only for the color preference using a different behavior (classical component). This test with a different behavior was performed as described previously (Brembs and Heisenberg, 2000). Briefly, the panorama of the fly is replaced with a new arena, containing four evenly spaced, identical vertical stripes. Each stripe denotes the center of a virtual 90° quadrant. A computer controlled electric motor rotates the arena such that its angular velocity is proportional to, but directed against the fly's yaw torque. The color of the illumination of the whole arena is changed whenever one of the virtual quadrant borders passes a point in front of the fly. During the 60s familiarization/reminder training, heat punishment is made contiguous with the color punished in the previous composite learning phase. During test, the heat is permanently switched off. Despite relying on yaw torque as the composite situation, this test for the generalization of the classical memory requires the animal to use a different motor

output than was used during composite training. While during composite training the animal had to constantly turn in one direction to keep arena illumination constant, in this flight-simulator-like situation, the animal has to fly straight to accomplish the same effect. Thus, any operant component learned during composite training would interfere with generalization of the classical component.

Data evaluation. The color or yaw torque domain preference of individual flies is calculated as the performance index: $PI=(t_a-t_b)/(t_a+t_b)$. During training periods, t_b indicates the time the fly is exposed to the heat and t_a the time without heat. During tests, t_a and t_b refer to the times when the fly chose the formerly (or subsequently) unpunished or punished situation, respectively.

Statistics. Individual PI's were tested for significance using a t-Test for single means against zero, following previous studies (Liu et al., 1999; Brembs and Hempel de Ibarra, 2006; Brembs and Wiener, 2006; Liu et al., 2006). All data are expressed as means ± SEM.

Results

Flies were first trained with operant behavior and classical stimuli equivalently predicting the heat and then tested in three different situations: (1) In a control situation where nothing has changed. (2) In a situation without the classical stimuli (by removing the color filters), leaving only the operant component. (3) In a situation where only the classical stimuli were present and the operant behavior controlling them exchanged to a novel one. Each test was performed without heat (Fig. 1).

Wildtype flies produce significant PIs for the control test (1)(Fig. 2a), but fail to show a significant learning score after composite training when tested without the classical predictor (2) (Fig. 2a), not even after a 60s reminder training (data not shown). Flies only impaired in classical learning (*rut* mutant flies) fail the composite control test (1), despite their intact operant learning system (Fig. 2b and Brembs and Plendl, 2008). When *rut* mutant flies are tested for the operant component after composite training (2), they show a significant performance index (Fig. 2b). Without initial classical learning, the test for generalization (3) is superfluous in *rut* flies and wildtype flies this generalization has already been shown (Brembs and Heisenberg, 2000). These data indicate that even if there were an operant memory which would lead to a significant learning score, the presence of classical stimuli inhibits access to this memory. Corroborating this notion, wildtype flies also fail a composite test with both predictors if they are trained with only the operant predictor and the classical

predictor (to which the flies are naïve) is added only during the test (unpublished observation).

These results suggest that the classical system somehow inhibits the operant system. To study the neural basis of this inhibition of operant learning, the UAS-GAL4 system was used to block synaptic output in a range of neuronal circuits by expressing the bacterial tetanus toxin light chain. Because of the role of the MB in generalization, (Liu et al., 1999; Brembs and Wiener, 2006), this prominent paired neuropil was the first target. All transgenic flies were first trained with equivalent operant and classical predictors and then tested in the three different situations as described above. Flies with impaired MB function can learn both the colors and to modulate their yaw torque (Wolf et al., 1998; Brembs and Wiener, 2006). Hence, it comes as no surprise that flies expressing tetanus toxin in 710 Kenyon-cells projecting to all MB-lobes (via P[GAL4] line mb247) could master the composite learning task composed of these two predictors (Fig. 3a). However, in contrast to the heterozygous parental control strains which reproduced the wildtype results in all three tests, flies with such blocked MB output do not generalize the classical memory to a novel behavior and show significant learning of the operant component (Fig. 3a, b). Apparently, flies without MB-function can learn the classical component, but nevertheless engage the operant component, which interferes with generalization.

To investigate which of the MB-lobes are responsible for the inhibition of operant learning in composite situations, transgenic flies with the P[GAL4] driver line 17D, which drives toxin expression in the mushroom-body α- and β-lobes, but not in the γ-lobes (Martin et al., 1998) were subjected to the same procedure. These flies show the same pattern of PIs as the flies expressing tetanus toxin in most Kenyon cells: significant PIs in the control and in the purely operant test and no significant score in the generalization test (Fig. 3c), conclusively tying the inhibition of the operant component to the MB. Moreover, I tentatively conclude that the MB γ-lobes are probably not involved in this process. The specificity of these effects was confirmed by using the P[GAL4] driver line c205 to constitutively express tetanus toxin in the F5 neurons in the fan-shaped body of the central complex. Flies in which the c205 line drives expression of a constitutively active G-Protein are defective in visual pattern discrimination learning (Liu et al., 2006). Nevertheless, these flies behaved similar to wildtype and genetic control flies (Fig. 3d).

Because of the interference with generalization and the apparent parallels to habit formation interfering with behavioral flexibility in other animals (Krakauer et al., 2006; Yin

and Knowlton, 2006), wildtype flies were subjected to an extended composite training regime (Fig. 3e). The hypothesis was that if learning of the operant behavior is analogous to habit formation, extended training should overcome the inhibition of operant learning mediated by the MB, lead to acquisition of the operant component and a failure to generalize, i.e., essentially a phenocopy of the flies with blocked MB output. Consistent with this hypothesis, flies which were trained with equivalent operant and classical predictors for twice the regular amount of time show the same pattern of PIs as the transgenic flies with impaired MB function: significant PIs in the control and in the operant test and no significant score in the generalization test (Fig. 3e).

Discussion

A wealth of knowledge has accumulated about the memory trace formed during classical olfactory conditioning in the MB of *Drosophila* (Gerber et al., 2004; Davis, 2005; Akalal et al., 2006). The current consensus posits that classical odor memory is laid down within the Kenyon cells. This is clearly not the case for visual learning, where the MB are not essential (Wolf et al., 1998; Brembs and Wiener, 2006). Instead, some visual memory appears to reside within the fan-shaped body of the central complex (Liu et al., 2006). For visual learning, the MB appear to stabilize classical memories against changes in the fly's situation. If the fly's sensory situation changes, this feature supports context generalization (Liu et al., 1999; Brembs and Wiener, 2006) and protects against sensory conflict (Tang and Guo, 2001; Zhang et al., 2007). If the fly's behavioral situation changes, this feature supports the form of generalization described here. From these results, the role of the MB for the stabilization of classical memories appears to be much more pervasive than previously imagined. The current data are consistent with the hypothesis that the MB perform a suppressive function allowing only certain neuronal events to take place, but not others. Such a function is also well in line with the current picture of the general insect MB function being largely inhibitory in nature (Huber, 1965; Wahdepuhl, 1983; Martin et al., 1998).

These insights allow for the first time to establish a mechanistic model of how operant and classical learning systems may interact in composite situations and which biological substrates mediate these processes (Fig. 4). The AC-dependent classical component suppresses acquisition of the PKC-dependent operant component via the MB. The operant system facilitates classical learning via still unknown, non-MB pathways (data not shown and Brembs and Heisenberg, 2000). This interaction leads to efficient learning, enables generalization and prevents premature habit formation. Habit formation after extended

training reveals the gate-keeping role of the MB, allowing only well-rehearsed behaviors to crystallize into habits.

It remains a tantalizing finding for all *Drosophila* learning and memory research that overtrained wildtype flies behave indistinguishably from flies with blocked MB output. This is reminiscent of vertebrate experiments, where the dorsal striatum and the hippocampus are viewed as competing learning systems with the dorsal striatum involved in skill-learning and the hippocampus in fact-learning (Yin and Knowlton, 2006). Short training is primarily processed by the hippocampus, while prolonged training recruits the dorsal striatum.

Combining the tools developed in the approach of localizing memory traces with the experimental separation of operant and classical components, *Drosophila* has now entered the stage where we can start to unravel not only where memories are stored, but also how basic neural subsystems interact to accomplish efficient learning in ethologically relevant situations, without compromising generalization or prematurely engaging habit-formation. Research on *Drosophila* has provided key insights into mechanisms of classical learning that are evolutionary conserved. The utility of this model system has now been extended to the study of complex learning situations comprising multiple, interacting memory systems on the behavioral, circuit and genetic level. These studies expand a growing body of literature that simultaneously engaged memory systems can act both cooperatively and antagonistically.

References

Akalal DB, Wilson CF, Zong L, Tanaka NK, Ito K, Davis RL (2006) Roles for Drosophila mushroom body neurons in olfactory learning and memory. Learn Mem 13:659-668.

Baier A, Wittek B, Brembs B (2002) Drosophila as a new model organism for the neurobiology of aggression? J Exp Biol 205:1233-1240.

Brand AH, Perrimon N (1993) Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118:401-415.

Brembs B (2008) Operant learning of *Drosophila* at the torque meter. JoVE 16.:http://www.jove.com/index/Details.stp?ID=731, doi: 710.3791/3731.

Brembs B, Heisenberg M (2000) The operant and the classical in conditioned orientation in *Drosophila melanogaster* at the flight simulator. Learn Mem 7:104-115.

Brembs B, Hempel de Ibarra N (2006) Different parameters support generalization and discrimination learning in *Drosophila* at the flight simulator. Learn Mem 13:629-637.

Brembs B, Wiener J (2006) Context generalization and occasion setting in *Drosophila* visual learning. Learn Mem 13:618-628.

Brembs B, Plendl W (2008) Double dissociation of PKC and AC manipulations on operant and classical learning in *Drosophila*. Curr Biol 18:1168-1117.

Brembs B, Baxter DA, Byrne JH (2004) Extending *in vitro* conditioning in *Aplysia* to analyze operant and classical processes in the same preparation. Learning and Memory 11:412-420.

Brembs B, Lorenzetti FD, Reyes FD, Baxter DA, Byrne JH (2002) Operant reward learning in Aplysia: neuronal correlates and mechanisms. Science 296:1706-1709.

Davis RL (2005) Olfactory memory formation in Drosophila: from molecular to systems neuroscience. Annu Rev Neurosci 28:275-302.

Gerber B, Tanimoto H, Heisenberg M (2004) An engram found? Evaluating the evidence from fruit flies. Curr Opin Neurobiol 14:737-744.

Gormezano I, Tait RW (1976) The Pavlovian analysis of instrumental conditioning. Pavlov J Biol Sci 11:37-55.

Götz KG (1964) Optomotorische Untersuchung des visuellen Systems einiger Augenmutanten der Fruchtfliege Drosophila. Kybernetik 2:77-92.

Guo A, Liu L, Xia S-Z, Feng C-H, Wolf R, Heisenberg M (1996) Conditioned visual flight orientation in *Drosophila*; Dependence on age, practice and diet. Learn Mem 3:49-59.

Heisenberg M, Wolf R, Brembs B (2001) Flexibility in a single behavioral variable of Drosophila. Learn Mem 8:1-10.

Huber F (1965) Brain controlled behavior in orthopterans. In: The physiology of the insect nervous system (Treherne J, Beament J, eds), pp 233-246. London: Academic Press.

Krakauer JW, Mazzoni P, Ghazizadeh A, Ravindran R, Shadmehr R (2006) Generalization of Motor Learning Depends on the History of Prior Action. PLoS Biology 4:e316.

Liu G, Seiler H, Wen A, Zars T, Ito K, Wolf R, Heisenberg M, Liu L (2006) Distinct memory traces for two visual features in the *Drosophila* brain. Nature 439:551-556.

Liu L, Wolf R, Ernst R, Heisenberg M (1999) Context generalization in *Drosophila* visual learning requires the mushroom bodies. Nature 400:753-756.

Lorenzetti FD, Baxter DA, Byrne JH (2008) Molecular Mechanisms Underlying a Cellular Analog of Operant Reward Learning. Neuron 59:815-828.

Lorenzetti FD, Mozzachiodi R, Baxter DA, Byrne JH (2006) Classical and operant conditioning differentially modify the intrinsic properties of an identified neuron. Nat Neurosci 9:17-29.

Martin J-R, Ernst R, Heisenberg M (1998) Mushroom Bodies Suppress Locomotor Activity in *Drosophila melanogaster*. Learn Mem 5:179-191.

Miller S, Konorski J (1928) Sur une forme particuliere des reflexes conditionnels. C r Soc Biol 99:1155-1157.

Ostlund SB, Balleine BW (2007) Orbitofrontal cortex mediates outcome encoding in pavlovian but not instrumental conditioning. J Neurosci 27:4819-4825.

Rescorla RA, Solomon RL (1967) Two-process learning theory: Relationships between Pavlovian conditioning and instrumental learning. Psychol Rev 74:151-182.

Rister J, Heisenberg M (2006) Distinct functions of neuronal synaptobrevin in developing and mature fly photoreceptors. J Neurobiol 66:1271-1284.

Sweeney ST, Broadie K, Keane J, Niemann H, O'Kane CJ (1995) Targeted expression of tetanus toxin light chain in Drosophila specifically eliminates synaptic transmission and causes behavioral defects. Neuron 14:341-351.

Tang S, Guo A (2001) Choice Behavior of Drosophila Facing Contradictory Visual Cues. Science 294:1543-1547.

Thum AS, Knapek S, Rister J, Dierichs-Schmitt E, Heisenberg M, Tanimoto H (2006) Differential potencies of effector genes in adult *Drosophila*. The Journal of Comparative Neurology 498:194-203.

Wahdepuhl M (1983) Control of grasshopper singing behavior by the brain responses to electrical stimulation. Z Tierpsychol 63:173-200.

Wolf R, Heisenberg M (1991) Basic organization of operant behavior as revealed in *Drosophila* flight orientation. J Comp Physiol [A] 169:699-705.

Wolf R, Wittig T, Liu L, Wustmann G, Eyding D, Heisenberg M (1998) *Drosophila* mushroom bodies are dispensable for visual, tactile and motor learning. Learn Mem 5:166-178.

Yin HH, Knowlton BJ (2006) The role of the basal ganglia in habit formation. Nat Rev Neurosci 7:464-476.

Zhang K, Guo JZ, Peng Y, Xi W, Guo A (2007) Dopamine-Mushroom Body Circuit Regulates Saliency-Based Decision-Making in *Drosophila*. Science 316:1901-1904.

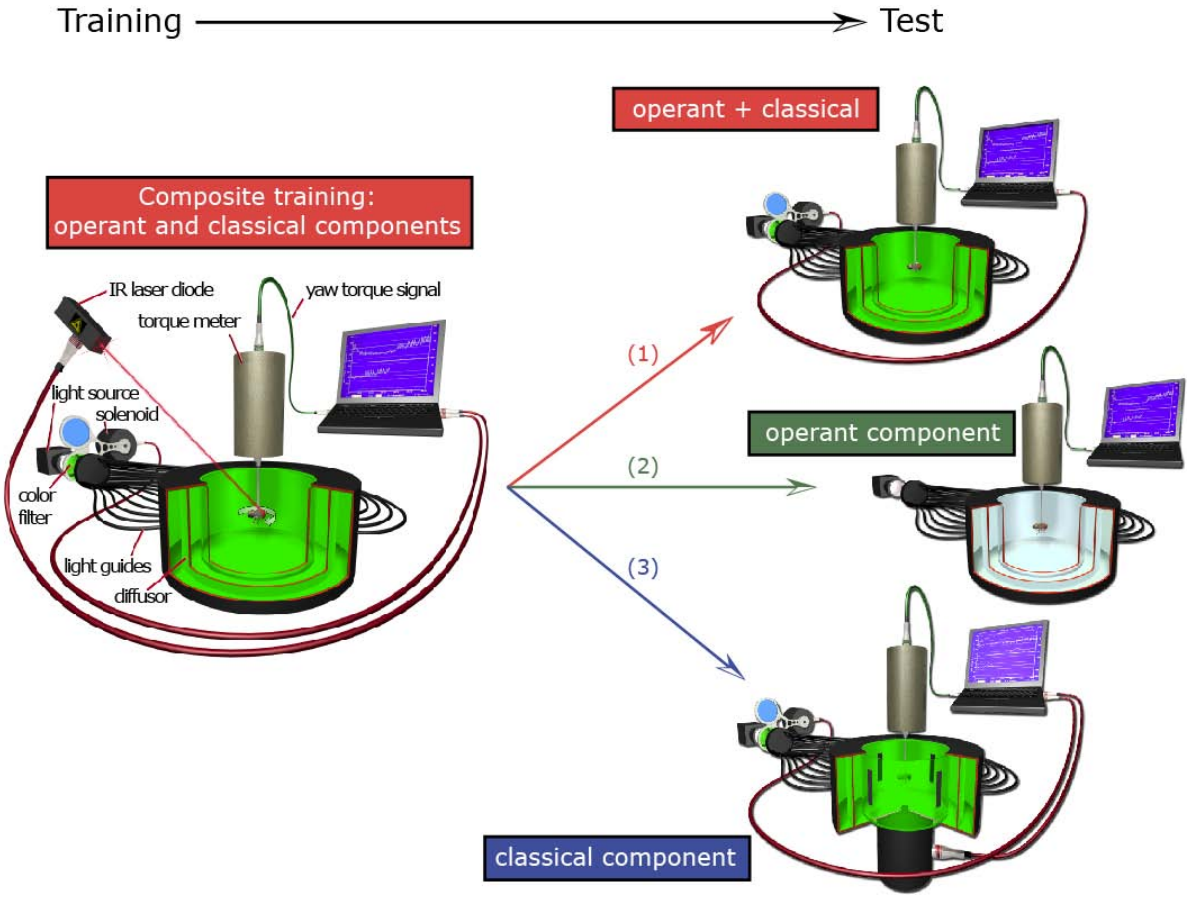


Figure 1: *Experimental design*.
During training, heat is used to simultaneously condition flies both to avoid turning to one direction (right or left; operant component) and one of two colors (blue or green, classical component). In a subsequent test without heat, the flies' spontaneous preference is recorded. One group of flies is tested in the same situation as during training (1). A second group of flies is tested for the operant component in isolation by removing the classical component (2). A third group of flies is tested for the classical component by replacing the operant behavior controlling the colors with a novel behavior (see Materials and Methods and Brembs, 2008 for details).

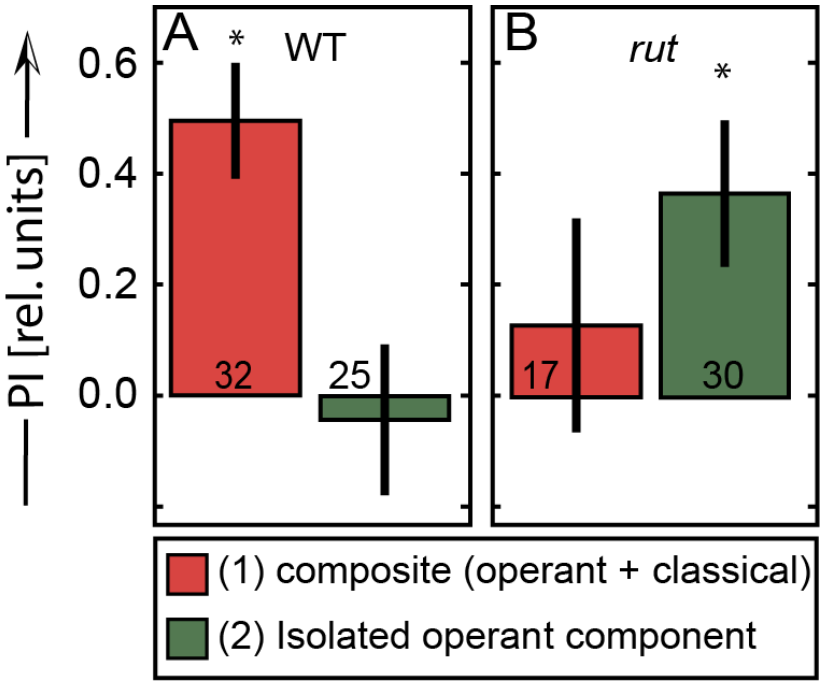


Figure 2: *The hierarchical interaction between operant and classical learning systems is brought about by an inhibition of operant learning.*
A. Significant composite learning in wildtype (WT) flies (t_{31}=5.1, p<0.001). After composite training, the score for the isolated operant component is not significant (t_{24}=-0.3, p<0.8) indicating inhibition of the operant system during acquisition.
B. Abolished composite learning *rut* mutant flies (t_{16}=0.7, p<0.5). After composite training, there remains a significant operant component in *rut* mutant flies (t_{29}=2.9, p<0.007) indicating inhibition of the operant system at the level of retrieval.
Numbers at bars – number of animals. * – significant difference from zero. Error bars are s.e.m. throughout.

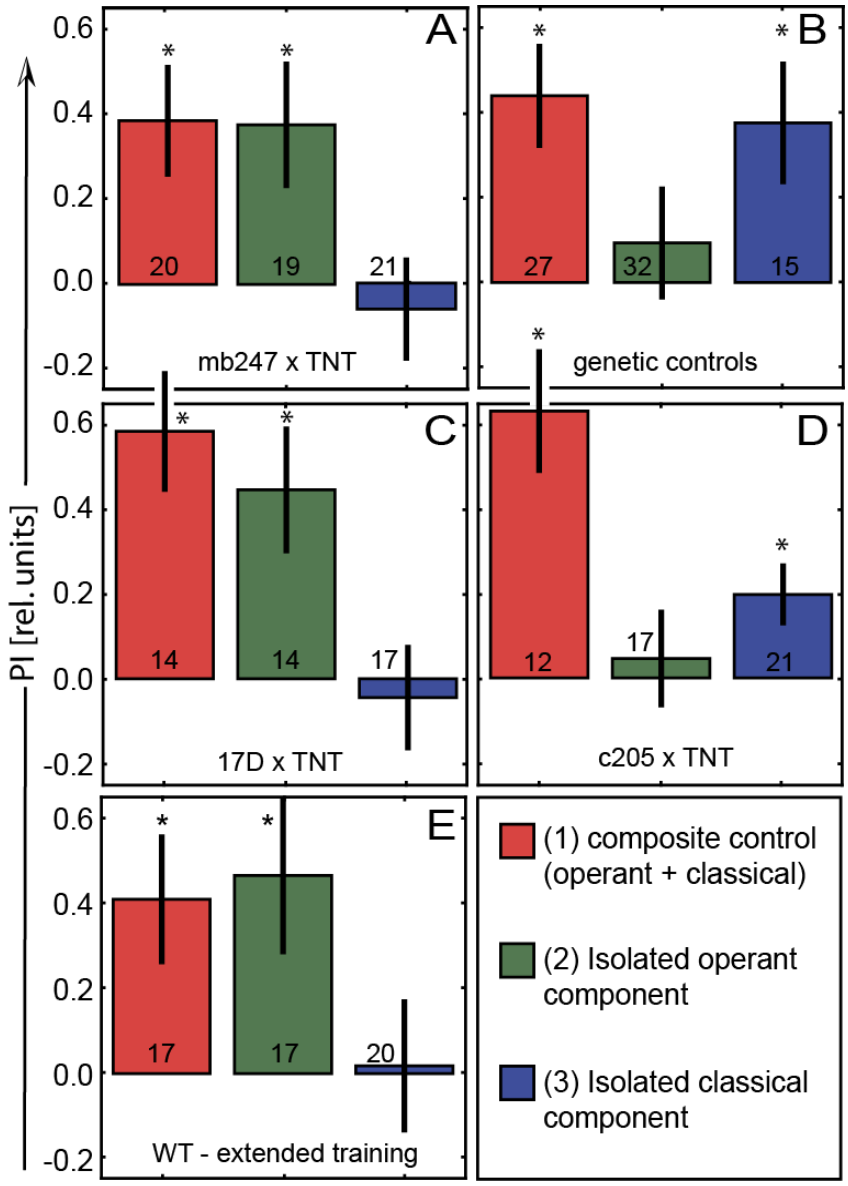


Figure 3: *The mushroom-body α and β lobes but not the γ lobes are necessary for inhibition of the operant component and generalization of classical memory.*

A. Flies with blocked MB output perform well in composite learning (red: t_{19}=3.1, p<0.01), but do not inhibit the operant component during composite training (green: t_{18}=2.6, p<0.05). Without inhibition of operant system, these transgenic flies are unable to generalize the isolated classical component to a novel behavior (blue: t_{20}=-0.5, p<0.6.).

B. The genetic control flies (the two heterozygote strains did not differ and were pooled) reproduce the wild-type results: significant composite learning (t_{26}=3.8, p<0.001), inhibition of the operant system (t_{31}=0.7, p<0.5) and successful generalization of the isolated classical component (t_{14}=2.7, p<0.05).

C. Flies with blocked output only from the α and β lobes of the MB mimic the flies expressing tetanus toxin in all MB lobes. They perform well in composite learning (t_{13}=4.3, p<0.001), do not inhibit the operant system (t_{13}=3.1, p<0.01) and do not generalize (t_{16}=-0.38, p<0.71).

D. Specificity of our mushroom-body effects is provided by expressing TNT in the fan-shaped body. These flies behave as wildtype and control heterozygote flies with significant composite learning (t_{11}=4.3, p<0.002) and inhibition of the operant system (t_{16}=0.4, p<0.7), which in turn allows for a successful generalization of the classical component to a novel behavior (t_{20}=2.7, p<0.014).

E. Extended training in wildtype flies constitutes a phenocopy of the transgenic animals (**A**). The longer training duration does not lead to an overtraining decrement (t_{16}=2.8, p<0.013). Testing for the operant component shows a release from the inhibition of operant learning (t_{16}=2.6, p<0.02). Without inhibition of the operant system, the flies are unable to generalize (t_{19}=0.1, p<0.91).

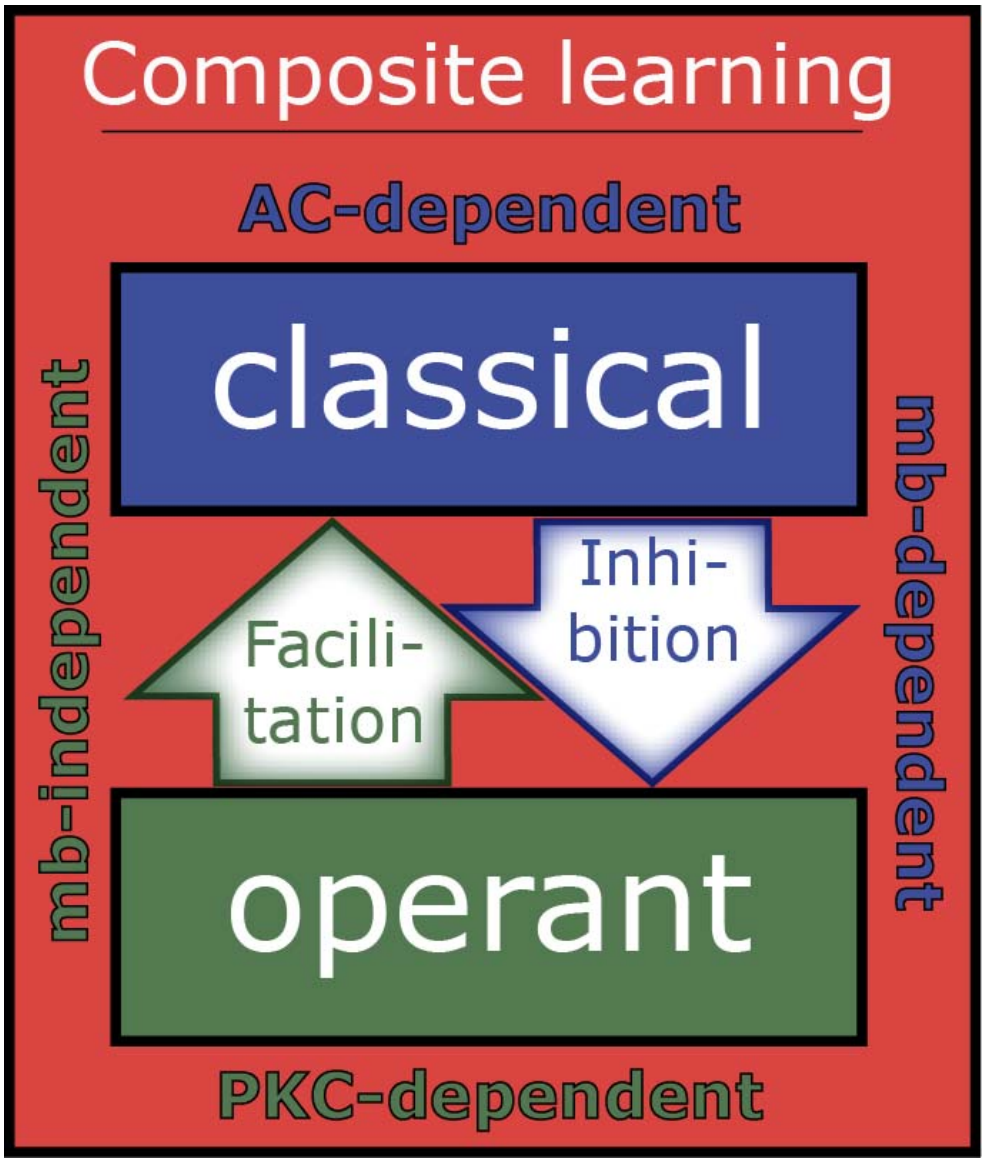


Figure 4: *Composite learning consists of two components with reciprocal, hierarchical interactions.*
The AC-dependent classical system inhibits PKC-dependent operant learning via the mushroom-bodies. Operant behavior controlling predictive stimuli facilitates learning about these stimuli by the classical system via unknown, non-mushroom-body pathways. These interactions lead to efficient learning, generalization and prevent premature habit-formation.

J. Neurogenetics, 2008, 1–7, iFirst article

ISSN: 0167-7063 print/1563-5260 online
DOI: 10.1080/01677060802471643

informa
healthcare

The Importance of Being Active

Björn Brembs
Freie Universität Berlin, Institut für Biologie–Neurobiologie, Berlin, Germany

Abstract: The successful stimulus-response approach to the organization of behavior has been the dominating paradigm for much of the psychology and neuroscience of the 20th century. Martin Heisenberg is a pioneer in championing the idea that all brains, even comparatively simple ones such as those of insects, instead operate according to output-input principles. Since the 1970s, his research produces evidence that the fruit fly, *Drosophila melanogaster*, is capable of spontaneous behavioral activity, and that the flies use it to control sensory input (i.e., operant behavior). Today, more and more evidence is accumulating also from fields outside of neuroscience that, indeed, one of the common, defining principles of all brains is this concept of operant behavior. Drawing from this evidence, it becomes clear that the conceptually simple process of generating activity and evaluating its consequences forms one of the fundamental cornerstones not only for all of our human nature, but also for our social coherence. This review recapitulates Heisenberg's most critical experiments and provides an overview over the current literature on the role of spontaneous activity in the ecology and evolution of brains. I conclude that spontaneous activity is both a necessary prerequisite and an inevitable consequence of evolution.

Keywords: spontaneous activity, intiating activity, evolution, brain, Heisenberg

Probably one of the—if not the best-understood sensory system is the fly visual system. Over the last 60 years or so, on all levels of complexity, the systems, the single cell and, more recently, even the molecular level, neuroscientists have developed an understanding that is unparalleled both in breadth and in depth. The groundwork was laid by the early works of Götz, Reichardt, and other colleagues in the tradition of biological cybernetics (Götz, 1964, 1965, 1968, 1972, 1977, 1980; Götz and Buchner, 1978; Götz et al., 1979; Kirschfeld and Reichardt, 1970; Poggio and Reichardt, 1973a, 1973b; Poggio and Reichardt, 1976a, 1976b; Reichardt, 1962; Reichardt and Poggio, 1975, 1976; Reichardt and Wenking, 1969; Reichardt, 1965; Wehrhahn and Reichardt, 1973). This tradition entailed to study the visual system with the tools of control theory. In principle, this meant interpreting such experiments as manipulating a complex input-output system. The idea behind this very successful black-box approach was to study the input-output relationships thoroughly enough to be able to construct a control model that could predict the motor output of the fly for any, even yet untested, visual input. One method of choice was often the so-called open-loop experiment, in which the tethered fly (Figure 1) received visual input while its motor output was recorded. Importantly, the motor output was not allowed to interfere with the presentation of the stimuli (i.e., the feedback loop between the animal's behavior and its environment was open). This was the time when the young geneticist, Martin Heisenberg, joined this exciting field after his postdoctoral period with Seymour Benzer at CalTech. Heisenberg's contributions to vision in *Drosophila* are covered elsewhere in this issue, so I will not go into any detail here. This input-output approach worked extremely well, and not only for biological cybernetics of visual guidance of insect flight. Many other areas of neurobiology, at the time, also thought of brains as input-output systems and prevented the behavior of their animals to interfere with their stimulus situation. In fact, so successful was this pervasive approach that until very recently, some neurobiologists still emphasized that ''brain function is ultimately best understood in terms of input/output transformations and how they are produced'' (Mauk, 2000). This was the dominant tradition in which Heisenberg was working when he moved to Würzburg to become the chair of the department of genetics in 1975.

In Würzburg, several discoveries prompted Heisenberg to radically change his view on brains. Three of these, in particular, were instrumental for his 180-degree turn. First was the observation that even without any variation in sensory input, the flies would produce varying motor output (Figure 2; Heisenberg and Wolf,

Received 20 August 2008; Accepted 10 September 2008.
Address correspondence to Björn Brembs, Freie Universität Berlin, Institut für Biologie–Neurobiologie, Königin Luise Str. 28–30, Berlin 14195, Germany. E-mail: bjoem@brembs.net

Figure 1. A *Drosophila* fruit fly, suspended at a copper hook, attached to the head and thorax of the fly by a small drop of glue.

1979). This finding flew in the face of every control theory thus far. A later mathematical analysis of the temporal structure of the flies' behavior in this situation (Maye et al., 2007) confirmed Heisenberg's interpretation that the variability in the behavior of the fly does not stem from a very noisy input-output system, but was generated actively by the fly, independently of any input (i.e., ''initiating activity''; Heisenberg, 1983, 1994). Importantly, Maye et al. (2007) found the same kind of variability in the behavior even when the flies were allowed to control their environment with the feedback loop closed. The second discovery was made with the double-mutant, *reduced optic lobes* (*rol*), *small optic lobes* (*sol*) (Figure 3). Freely walking or flying wild-type flies, in a visually structured rotating environment, have a tendency to turn with the direction of the movement. The *rol sol* double mutant flies still show phototaxis, but are completely devoid of any such directed ''optomotor response.'' The optomotor response was thought to be critical for stabilizing the animal in flight, and thus *rol sol* flies were expected to lack the capacity to use moving visual stimuli for course control and thus should show unstable flight. However, in experiments with tethered flies where the feedback loop between turning behavior and horizontal rotation of the environment was closed, *rol sol* mutant flies were able to stabilize their flight with respect to visual landmarks and fly straight (i.e., establish optomotor balance; Wolf & Heisenberg, 1986). The interpretation was that *rol sol* mutant flies are motion sensitive but lacked sensitivity to the direction of motion. This was demonstrated by performing the third critical experiment (Figure 4). After inversion of the feedback loop between behavior and environment, such that attempted left turns lead to a left turn of the environment and thus the visual impression of a right turn, *rol sol* mutants did not require any more time

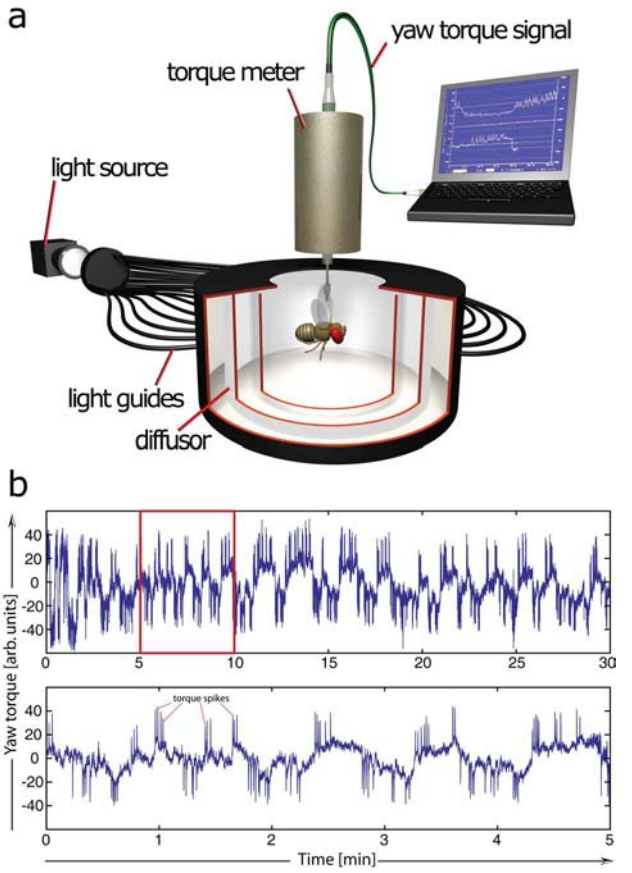


Figure 2. Measuring fruit fly spontaneity in a uniform, constant environment. (**A**) The fly is flying stationarily in a cylindrical arena homogeneously illuminated from behind. The fly's tendency to perform left or right turns (yaw torque) is measured continuously and fed into the computer. (**B**) Example of yaw torque data. Lower trace is a 5-minute enlargement of the 30-minute upper trace. Variability in two components of the behavior can be observed: slow baseline fluctuations and fast, superimposed torque spikes. Torque spikes correspond to body saccades in free flight.

to stabilize their flight and fly straight than when then loop was closed ''correctly.'' The conclusion that flies are actively initiating activity in order to ''try out'' which motor output controls the environment was confirmed when wild-type flies were subjected to this ''inverting goggles'' experiment. Even wild-type flies, with their optomotor response intact, eventually learned to use turning maneuvers of the ''opposite'' direction to control flight, that is, left-turning maneuvers for the visual impression of right turns and vice versa (Heisenberg and Wolf, 1984; Wolf et al., 1992). These three experiments attacked contemporary control theory from two ends: not only was output not predictable from input, but eliminating the open-loop response or inverting its direction could be compensated by plasticity in the system, such that it would still perform the function in question. Not only were the open loop situations an

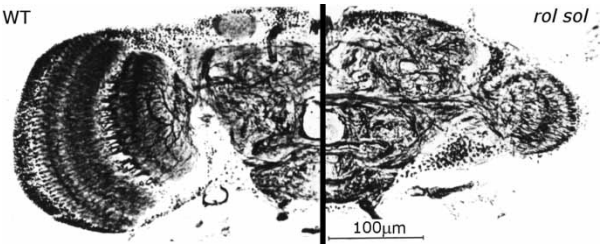


Figure 3. Frontal sections of wild-type (WT) and *rol sol* mutant brains. In *rol sol* flies, the optic lobes have about 12% of the wild-type volume. The remaining structures are retinotopically organized with the normal number of ommatidia in the eye and columns in the neuropil. Images courtesy of Martin Heisenberg.

inadequate experimental approach, but the input-output assumption itself proved inadequate as a theoretical construct for understanding even a fly brain. These experiments prompted Heisenberg to abandon this pervasive, successful approach and pursue a radically different research direction: how animals use their capacity for initiating output to control their sensory input. Today, 30 years later, more and more evidence—not only from neuroscience—is accumulating that while computing input-output relations may be an important feature of

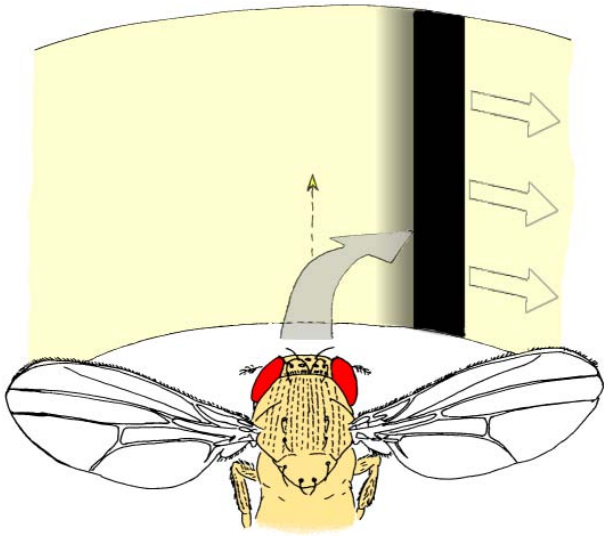


Figure 4. ''Inverting goggles'' experiment. Whenever the tethered fly attempts a turning maneuver, the fly's visual panorama is rotated in the same direction. In the depicted example, a right-turning maneuver leads to a rotation of the panorama to the right. In this situation, any attempts of the fly to follow the stripe will lead to a catastrophic feedback of increasing speed of the stripe and yaw torque of the fly in the same direction. Nevertheless, flies learn to generate turning maneuvers in the opposite direction in order to establish a zero net rotation of the stripe (optomotor balance). Fly drawing courtesy of Reinhard Wolf.

brains, computing such output-input relations (e.g., in so-called forward models; Webb, 2004) is probably equally, if not more, fundamental to the organization of behavior. In the remainder of this paper, I will try to provide a short sketch of the literature today that is supporting Heisenberg's early insight.

If we allow ourselves to anthropomorphize, Heisenberg's observation that flies with a constant stimulus situation still produce variable behaviors may not be all that surprising. We have all experienced how difficult it is to stay absolutely still, maybe even how horrible it feels to be forced not to move. We want to move and we want to decide what body part to move when and where the movement should go. We all feel the very basic notion that we possess a certain flexibility in our choices (Montague, 2008). Bereaving humans of such freedom is frequently used as punishment, and the bereft do invariably perceive this limited freedom as undesirable. This experience of freedom is an important characteristic of what it is like to be human. It stems, in part, from our ability to behave variably. Voltaire expressed this intuition in saying, ''Liberty then is only and can be only the power to do what one will'' (Voltaire, 1752/1924). However the concept that we can decide to behave differently, even under identical circumstances, underlies not only our justice systems, but our electoral systems, educational systems, parenting, and, basically, all other social systems also presuppose behavioral variability and at least a certain degree of freedom of choice. Games and sports would be predictable and boring without our ability of constantly changing our behavior in always the same settings. Faced with novel situations, humans and most animals increase their behavioral variability (Bunzeck and Duzel, 2006; Roberts and Gharib, 2006; Shahan and Chase, 2002). Animals even vary their behavior when a more stereotyped behavior would be more efficient (Krechevsky, 1937). Inasmuch as behavioral variability between individuals has genetic components, it is a crucial factor of niche exploitation in evolution. Moreover, behavioral variability within individuals has been shown to be ecologically advantageous in game theoretical studies (Brembs, 1996; Glimcher, 2003, 2005; Glimcher and Rustichini, 2004; McNamara et al., 2004; Platt, 2004), in pursuit-evasion contests such as predator-prey interactions (''protean strategy'') (Driver and Humphries, 1988; Grobstein, 1994; Miller, 1997; Shultz and Dunbar, 2006), in exploration and foraging (Belanger and Willis, 1996; Viswanathan et al., 1999), in mobbing attack patterns by birds, and in the variation of male songbirds' songs (Neuringer, 2004). Clearly, invariable behavior will be exploited (Jablonski and Strausfeld, 2000, 2001; Miller, 1997) and leaves an organism helpless in unpredictable situations (Greenspan, 2005; Heisenberg, 1994).

Thus, competitive success and evolutionary fitness of all ambulatory organisms rely critically on intact behavioral variability as an adaptive brain function. But, relative freedom from environmental contingencies is a necessary, but most often not a sufficient, criterion for such accomplishments. Tightly connected to the ability to produce variable behavior is the ability to use the effects of these behaviors to control the environment. The incoming stream of sensory information is noisy and fluctuates for any number of reasons. Any covariance between the behavioral variations and those of sensory input indicates that the latter are consequences of the behavior and can thus be controlled be the animal (Bays et al., 2006; Wegner, 2002). Every animal relies heavily on this on-line detection system for when the animal itself is the reason for any environmental fluctuation. This function is so paramount that we humans express our delight over control of our environment (including other people) already as children, by, for example, shrieking in excitement when Dad jumps after a ''boo'' or proudly presenting Mom with ''look what I can do!''. Later, children find pleasure in building airplane models, become carpenters with a delight for shaping wood, artists feeling gratified creating art out of the simplest materials, musicians enjoying mastering their instrument to perfection, athletes, scientists, engineers, managers, or cunning politicians. Using trial and error, we have shaped our world from caves to skyscrapers, from horses to jet planes, from spears to hydrogen bombs. Cultural or religious rituals (e.g., rain dance) and superstition may have developed as a means to create a feeling of control where, ultimately, there is none. Clearly, behaving flexibly in order to control our environment is at the heart of human nature and probably affects more aspects of our daily lives than any other brain function. So essential is such functioning that even very simple brains possess it. Even *Drosophila* prefers a situation in which it controls its environment over one where it does not. If certain flight directions are experimentally superimposed with uncontrollable visual movements, flies quickly avoid such directions and fly only in areas of full control (Heisenberg et al., 2001). This experiment demonstrates that control over environmental stimuli is inherently rewarding already for numerically simple, but very likely also for all other, brains. The same experiment also helps to understand how the *rol sol* mutants managed to fly straight: The flies spontaneously varied their motor output (''trying out'') until they could control absolute movement (i.e., independently of direction) in their environment. Obviously, *rol sol* mutants are using an operant strategy to control their stimulus situation (Wolf and Heisenberg, 1986). The same strategy also must underlie the capacity of wild-type flies to master the ''inverting goggles'' experiment.

By detecting what component of the sensory stream is controlled by our own actions, operant behavior also underlies the distinction between observing and doing (i.e., differentiating between self and non-self). It is thought that one of the main principles behind operant behavior is the so-called reafference principle (Todorov, 2004; von Holst and Mittelstaedt, 1950; Webb, 2004). We compare our behavioral output (efference) with incoming sensory input (afference) to detect when we are the ones authoring environmental change (Bays et al., 2006; Wegner, 2002; Wolf and Heisenberg, 1991). One almost iconographic example of such behavior is to perform various spontaneous movements in front of a mirror to detect whether it is us we are perceiving. Even animals perform such movements in front of a mirror (Plotnik et al., 2006; Prior et al., 2008; Reiss and Marino, 2001). This automatic detection mechanism explains why we cannot tickle ourselves (Bays et al., 2006), why we perceive a stable visual world despite our frequent quick, or saccadic, eye movements (Sommer and Wurtz, 2006), and is reflected in different brain-activation patterns between self-generated and exogenous visual stimulation (Matsuzawa et al., 2005). It is thought that the detection is accomplished via an efference copy (or corollary discharge) of the motor command, which is compared to incoming afferent signals to distinguish re- from ex-afference. Such a differentiation has been implied to demonstrate causal reasoning in rats (Blaisdell et al., 2006; Clayton and Dickinson, 2006; Waldmann et al., 2006). Even robots can use such ''self-modeling'' to generate a continuously updated model of themselves and their environment (Bongard et al., 2006). The brain, then, uses this model to predict the sensory consequences of behavior, and the integration of this prediction with the actual sensory information is used to produce an estimate of sensory space that is enhanced over predictions from either ex- or reafferent stimulation alone (Vaziri et al., 2006). This effect of operant enhancement of sensory cues can be observed also in the fruit fly (Brembs and Plendl, 2008; Heisenberg et al., 2001), monkey (Kornell and Terrace, 2007), human (James, 1890; Slamecka and Graf, 1978), and robot (Gutnisky and Zanutto, 2004b) learning and may explain why starlings, but not tamarin monkeys, can recognize patterns defined by so-called recursive grammar (Marcus, 2006). Such control of sensory input has often been termed ''goal-directed'' behavior or action. At its basis lies the capacity to generate spontaneous variability: initiating activity. This perspective provides an intuitive understanding of the rewarding properties of being in control of the environment. Setting and obtaining goals is inherently rewarding (Kim et al., 2006). This reward ensures that individuals always actively strive to control. Expecting sensory feedback signals can go so far that willing to move a limb can lead to the illusion of limb movement, even if

none occurred (Gandevia et al., 2006). One may also say that we so want our actions to have an effect that we sometimes develop a bad conscience even when we have not done anything wrong.

At the same time, by controlling the environmental input by using operant feedback loops, individuals exert their effect not only on themselves, but their survival and procreation in the environment they shape for themselves directly affects evolution. This has been shown in the field, for example, for western bluebirds, which dissociate into different niches according to their level of aggression (Duckworth, 2006). In humans, such mechanisms have been proposed to explain otherwise hard-to-understand phenomena, such as high IQ heritability estimates and associated paradoxa (i.e., increasing IQ heritability with age/experience and the ''Flynn effect'' of increasing IQ over generations) (Dickens and Flynn, 2001; Toga and Thompson, 2005). Another good example is the evolution of brain size. Most inter- and intraspecific interactions can be conceptualized as pursuit-evasion contests (e.g., predator/prey, male/female, dominant/subordinate, etc.). There are two reports on such contests leading to increased brain size. The first details how small-brained prey are more likely to be caught by predators, presumably because their capacity for behavioral variability is also smaller (Shultz and Dunbar, 2006). The second shows that the largest relative brain sizes among primate species are associated with monogamous mating systems, raising the suspicion that unpredictable mating strategies are the most successful ones in monogamous species (Schillaci, 2006). Other research in birds ties the evolution of brain size both to behavioral variability and migration: birds with larger brains are both more likely to be sedentary and cope better in novel environments. The hypothesis here is that a sedentary lifestyle in seasonally changing habitats requires significant behavioral flexibility. Operant feedback provides flexible birds with more resources, which enable them to support larger brains, which, in turn, generate more behavioral variability: Brain size and behavioral flexibility coevolved to outcompete other, smaller-brained birds that migrate in order to survive (Pravosudov et al., 2007; Sol et al., 2005a,b). Thus, the interdependence of brain size, the level of behavioral variability it provides, and the energy supply by which it is constrained are starting to unravel.

CONCLUSIONS

With this short overview, I hope to have shown that without initiating activity, there would not be any brains for us to study. Such spontaneous activity is both a necessary prerequisite and an inevitable consequence of evolution. Ultimately, the conceptually simple process of generating activity and evaluating its consequences forms one of the fundamental cornerstones not only for all of our human nature, but also for our social coherence: human nature as described in planning, willing, and controlling our behavior (Frith et al., 1991; Knight et al., 1995; Lezak, 1995; Owen, 1997; Wegner, 2002) and our social coherence, as based on cooperation (Gutnisky and Zanutto, 2004a; McNamara et al., 2004; Sanabria et al., 2003). Martin Heisenberg is a visionary and a pioneer in the neurobiological study of these and related neural processes. Only recently has his view of the primarily active nature of brains started to gain a more widespread acceptance among biologists.

ACKNOWLEDGMENTS

The author would like to thank Martin Heisenberg for his continued support and encouragement, for sharing his knowledge, experience, and wisdom, and for his way of leading by example.

Declaration of interest: The author reports no conflict of interest. The author alone is responsible for the content and writing of this paper.

REFERENCES

Bays, P. M., Flanagan, J. R. & Wolpert, D. M. (2006). Attenuation of self-generated tactile sensations is predictive, not postdictive. *PLoS Biol*, *4*(2), e28.

Belanger, J. H. & Willis, M. A. (1996). Adaptive control of odor-guided locomotion: behavioral flexibility as an antidote to environmental unpredictability. *Adapt Behav*, *4*(3–4), 217–253.

Blaisdell, A. P., Sawa, K., Leising, K. J. & Waldmann, M. R. (2006). Causal reasoning in rats. *Science*, *311*(5763), 1020–1022.

Bongard, J., Zykov, V. & Lipson, H. (2006). Resilient machines through continuous self-modeling. *Science*, *314*(5802), 1118–1121.

Brembs, B. (1996). Chaos, cheating, and cooperation: potential solutions to the Prisoner's Dilemma. *Oikos*, *76*(1), 14–24.

Brembs, B. & Plendl, W. (2008). Double dissociation of PKC and AC manipulations on operant and classical learning in *Drosophila*. *Curr Biol*, *18*(15), 1168–1117.

Bunzeck, N. & Duzel, E. (2006). Absolute coding of stimulus novelty in the human substantia nigra/VTA. *Neuron*, *51*(3), 369–379.

Clayton, N. & Dickinson, A. (2006). Rational rats. *Nat Neurosci*, *9*(4), 472–474.

Dickens, W. T. & Flynn, J. R. (2001). Heritability estimates versus large environmental effects: the IQ paradox resolved. *Psychol Rev*, *108*(2), 346–369.

Driver, P. M. & Humphries, N. (1988). *Protean behavior: the biology of unpredictability*. Oxford, England: Oxford University Press.

Duckworth, R. (2006). Aggressive behaviour affects selection on morphology by influencing settlement patterns in a passerine bird. *Proc. R. Soc. Lond. B*, *273*(1595): 1789–95.

Frith, C. D., Friston, K., Liddle, P. F. & Frackowiak, R. S. (1991). Willed action and the prefrontal cortex in man: a study with PET. *Proc R Soc Lond B Biol Sci*, *244*(1311), 241–246.

Gandevia, S. C., Smith, J. L., Crawford, M., Proske, U. & Taylor, J. L. (2006). Motor commands contribute to human position sense. *J Physiol (Lond)*, *571*(3), 703–710.

Glimcher, P. (2003). *Decisions, Uncertainty, and the Brain: the Science of Neuroeconomics*. Cambridge, Massachusetts, USA: MIT.

Glimcher, P. W. (2005). Indeterminacy in brain and behavior. *Annu Rev Psychol*, *56*, 25–56.

Glimcher, P. W. & Rustichini, A. (2004). Neuroeconomics: the consilience of brain and decision. *Science*, *306*(5695), 447–452.

Götz, K. G. (1964). Optomoter studies of the visual system of several eye mutants of the fruit fly. *Drosophila*. *Kybernetik*, *2*, 77–92.

Götz, K. G (1965). The optical transfer properties of the complex eyes of *Drosophila*. *Kybernetik*, *2*, 215–221.

Götz, K. G. (1968). Flight control in *Drosophila* by visual perception of motion. *Kybernetik, 4*, 199–208.

Götz, K. G. (1972). Principles of optomotor reactions in insects. Bibl Ophthalmol, *82*, 251–259.

Götz, K. G. (1980). Visual guidance in *Drosophila*. Basic Life Sci, *16*, 391–407.

Götz, K. G., & Buchner, E. (1978). Evidence for one-way movement detection in the visual system of *Drosophila*. Biol Cybern, *31*, 243–248.

Götz, K. G., Hengstenberg, B., & Biesinger, R. (1979). Optomotor control of wingbeat and body posture in *Drosophila*. *Biol Cybern, 35*, 101–112.

Greenspan, R. J. (2005). No critter left behind: an invertebrate renaissance. *Curr Biol*, *15*(17), R671–R672.

Grobstein, P. (1994). Variability in behavior and the nervous system. In Ramachandran, V. S. (Ed.), *Encyclopedia of Human Behavior* (Vol. 4, pp. 447–458). New York: Academic Press.

Gutnisky, D. A. & Zanutto, B. S. (2004a). Cooperation in the iterated Prisoner's Dilemma is learned by operant conditioning mechanisms. *Artif Life*, *10*(4), 433–461.

Gutnisky, D. A. & Zanutto, B. S. (2004b). Learning obstacle avoidance with an operant behavior model. *Artif Life*, *10*(1), 65–81.

Heisenberg, M. (1983). Initiale aktivität und willkürverhalten bei tieren. *Naturwissenschaften, 20*, 70–78.

Heisenberg, M. (1994). Voluntariness (willkürfähigkeit) and the general organization of behavior. *Life Sci Res Rep*, *55*, 147–156.

Heisenberg, M. & Wolf, R. (1979). On the fine structure of yaw torque in visual flight orientation of drosophila-melanogaster. *J Comp Physiol A Sens Neural Behav Physiol*, *130*(2), 113–130.

Heisenberg, M. & Wolf, R. (1984). *Vision in Drosophila. Genetics of Microbehavior*. Berlin, Heidelberg, New York, Tokyo: Springer.

Heisenberg, M., Wolf, R. & Brembs, B. (2001). Flexibility in a single behavioral variable of *Drosophila*. *Learn Mem*, *8*(1), 1–10.

Jablonski, P. G. & Strausfeld, N. J. (2000). Exploitation of an ancient escape circuit by an avian predator: prey sensitivity to model predator display in the field. *Brain Behav Evol*, *56*(2), 94–106.

Jablonski, P. G. & Strausfeld, N. J. (2001). Exploitation of an ancient escape circuit by an avian predator: relationships between taxon-specific prey escape circuits and the sensitivity to visual cues from the predator. *Brain Behav Evol*, *58*(4), 218–240.

James, W. (1890). *The Principles of Psychology*. New York: Holt.

Kim, H., Shimojo, S. & O'Doherty, J. P. (2006). Is avoiding an aversive outcome rewarding? Neural substrates of avoidance learning in the human brain. *PLoS Biol*, *4*(8), 233.

Kirschfeld, K. & Reichardt, W. (1970). Optomotor experiments on Musca with linearly polarized light]. *Z Naturforsch B*, *25*(2), 228.

Knight, R. T, Grabowecky, M. F, & Scabini, D. (1995). Role of human prefrontal cortex in attention control. Adv Neurol, *66*, 21–34; discussion 34–26.

Kornell, N. & Terrace, H. S. (2007). The generation effect in monkeys. *Psychol Sci*, *18*(8), 682–685.

Krechevsky, I. (1937). Brain mechanisms and variability II. Variability where no learning is involved. *J Compar Physiol Psychol*, *23*, 139–160.

Lezak, M. D. (1995). *Neuropsychological Assessment* (3rd edn). New York: Oxford University Press.

Marcus, G. F. (2006). Language: startling starlings. *Nature*, *440*(7088), 1117–1118.

Matsuzawa, M., Matsuo, K., Sugio, T., Kato, C. & Nakai, T. (2005). Temporal relationship between action and visual outcome modulates brain activation: an fMRI study. *Magn Reson Med Sci*, *4*(3), 115–121.

Mauk, M. D. (2000). The potential effectiveness of simulations versus phenomenological models. *Nat Neurosci*, *3*(7), 649–651.

Maye, A., Hsieh, C.-H., Sugihara, G. & Brembs, B. (2007). Order in spontaneous behavior. *PLoS One*, *2*, e443.

McNamara, J. M., Barta, Z. & Houston, A. I. (2004). Variation in behaviour promotes cooperation in the Prisoner's Dilemma game. *Nature*, *428*(6984), 745–748.

Miller, G. F. (1997). Protean primates: The evolution of adaptive unpredictability in competition and courtship. In Whiten, A., & Byrne, R. W. (Eds.), *Machiavellian Intelligence II: Extensions and Evaluations* (pp. 312–340). Cambridge: Cambridge University Press.

Montague, P. R. (2008). Free will. *Curr Biol*, *18*(14), R584–R585.

Neuringer, A. (2004). Reinforced variability in animals and people: implications for adaptive action. *Am Psychol*, *59*(9), 891–906.

Owen, A. M. (1997). Cognitive planning in humans: neuropsychological, neuroanatomical, and neuropharmacological perspectives. *Prog Neurobiol*, *53*(4), 431–450.

Platt, M. L. (2004). Unpredictable primates and prefrontal cortex. *Nat Neurosci*, *7*(4), 319–320.

Plotnik, J. M., de Waal, F. B. M. & Reiss, D. (2006). Self-recognition in an Asian elephant. *PNAS*, *103*(45), 17053–17057.

Poggio, T. & Reichardt, W. (1973a). Considerations on models of movement detection. *Kybernetik*, *13*(4), 223–227.

Poggio, T. & Reichardt, W. (1973b). A theory of the pattern induced flight orientation of the fly Musca domestica. *Kybernetik*, *12*(4), 185–203.

Poggio, T. & Reichardt, W. (1976a). Nonlinear interactions underlying visual orientation behavior of the fly. *Cold Spring Harb Symp Quant Biol*, *40*, 635–645.

Poggio, T. & Reichardt, W. (1976b). Visual control of orientation behaviour in the fly. Part II. Towards the underlying neural interactions. *Q Rev Biophys*, *9*(3), 377–438.

Pravosudov, V. V., Sanford, K. & Hahn, T. P. (2007). On the evolution of brain size in relation to migratory behaviour in birds. *Anim Behav*, *73*(3), 535–539.

Prior, H., Schwarz, A. & Güntürkün, O. (2008). Mirror-induced behavior in the magpie (Pica pica): evidence of self-recognition. *PLoS Biol*, *6*(8), 202.

Reichardt, W. (1962). Nervous integration in the facet eye. *Biophys J*, *2*, 121–143.

Reichardt, W. & Poggio, T. (1975). A theory of the pattern induced flight orientation of the fly Musca domestica II. *Biol Cybern*, *18*(2), 69–80.

Reichardt, W, & Poggio, T. (1976). Visual control of orientation behaviour in the fly. Part I. A quantitative analysis. Q Rev Biophys, *9*(3), 311–375, 428–338.

Reichardt, W. & Wenking, H. (1969). Optical detection and fixation of objects by fixed flying flies. *Naturwissenschaften*, *56*(8), 424–425.

Reichardt, W. E. (1965). Quantum sensitivity of light receptors in the compound eye of the fly Musca. *Cold Spring Harb Symp Quant Biol*, *30*, 505–515.

Reiss, D. & Marino, L. (2001). Mirror self-recognition in the bottlenose dolphin: a case of cognitive convergence. *PNAS*, *98*(10), 5937–5942.

Roberts, S. & Gharib, A. (2006). Variation of bar-press duration: where do new responses come from? *Behav Proc*, *72*(3), 215–223.

Sanabria, F., Baker, F. & Rachlin, H. (2003). Learning by pigeons playing against tit-for-tat in an operant Prisoner's Dilemma. *Learn Behav*, *31*(4), 318–331.

Schillaci, M. A. (2006). Sexual Selection and the evolution of brain size in primates. *PLoS ONE*, *1*(1), 62.

Shahan, T. A. & Chase, P. N. (2002). Novelty, stimulus control, and operant variability. *Behav Anal*, *25*(2), 175–190.

Shultz, S. & Dunbar, R. (2006). Chimpanzee and felid diet composition is influenced by prey brain size. *Biol Lett*, *2*(4), 505–508.

Slamecka, N. J. & Graf, P. (1978). Generation effect—delineation of a phenomenon. *J Exp Psychol Hum Learn Mem*, *4*(6), 592–604.

Sol, D., Duncan, R. P., Blackburn, T. M., Cassey, P. & Lefebvre, L. (2005a). Big brains, enhanced cognition, and response of birds to novel environments. *Proc Natl Acad Sci U S A*, *102*(15), 5460–5465.

Sol, D., Lefebvre, L. & Rodriguez-Teijeiro, J. (2005b). Brain size, innovative propensity, and migratory behaviour in temperate Palaearctic birds. *Proc Royal Soc B Biol Sci*, *272*(1571), 1433–1441.

Sommer, M. A., & Wurtz, R. H. (2006). Influence of the thalamus on spatial visual processing in frontal cortex. *Nature*, 444: 374–377.

Todorov, E. (2004). Optimality principles in sensorimotor control. *Nat Neurosci*, *7*(9), 907–915.

Toga, A. W. & Thompson, P. M. (2005). Genetics of brain structure and intelligence. *Annu Rev Neurosci*, *28*, 1–23.

Vaziri, S., Diedrichsen, J. & Shadmehr, R. (2006). Why does the brain predict sensory consequences of oculomotor commands? Optimal integration of the predicted and the actual sensory feedback. *J Neurosci*, *26*(16), 4188–4197.

Viswanathan, G. M., Buldyrev, S. V., Havlin, S., da Luz, M. G., Raposo, E. P. & Stanley, H. E. (1999). Optimizing the success of random searches. *Nature*, *401*(6756), 911–914.

Voltaire. (1752/1924). Voltaire's Philosophical Dictionary (H. I. Wolf, Trans.). New York: Knopf.

von Holst, E., & Mittelstaedt, H. (1950). Das reafferenzprinzip. Wechselwirkungen zwischen zentralnervensystem und peripherie. *Naturwissenschaften, 37*, 464–476.

Waldmann, M. R., Hagmayer, Y. & Blaisdell, A. P. (2006). Beyond the information given: causal models in learning and reasoning. *Curr Direct Psychol Sci*, *15*(6), 307–311.

Webb, B. (2004). Neural mechanisms for prediction: do insects have forward models? *Trends Neurosci*, *27*(5), 278–282.

Wegner, D. M. (2002). *The Illusion of Conscious Will*. Boston: Bradford Books/MIT press.

Wehrhahn, C. & Reichardt, W. (1973). Visual orientation of the fly Musca domestica towards a horizontal stripe. *Naturwissenschaften*, *60*(4), 203–204.

Wolf, R. & Heisenberg, M. (1986). Visual orientation in motion-blind flies is an operant behavior. *Nature*, *323*(6084), 154–156.

Wolf, R. & Heisenberg, M. (1991). Basic organization of operant behavior as revealed in *Drosophila* flight orientation. *J Compar Physiol A Sens Neur Behav Physiol*, *169*, 699–705.

Wolf, R., Voss, A., Hein, S. & Heisenberg, M. (1992). Can a fly ride a bicycle? Discussion on natural and artificial low-level seeing systems. *Philos Transact Royal Soc London Series B Biol Sci*, *337*(1281), 261–269.