The neuronal basis of operant self-learning in *Drosophila melanogaster*

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Abstract

Speech is a key feature distinguishing humans from any other species. *FoxP2* was discovered as a gene involved in speech production in humans. The operant process of vocal learning shares key characteristics with the torque learning of *Drosophila* in the flight simulator: an initial variable behaviour gets narrowed down via an operant feedback loop. *FoxP* mutant flies are specifically impaired in an operant self-learning task. Here, the flies don't have any externals cues about the experimental outcome, only their own behaviour. To understand more about the underlying mechanism, several *FoxP* related manipulation were performed.

We showed that *FoxP* is not required for operant self-learning in adult flies but is needed for maintaining learning ability. No single brain area could be identified, were intact *FoxP* expression is necessary for this learning typ. We showed that *PKC53e* is not involved in this learning task. On the other hand, we found that *aPKC* is a relevant gene for this learning behaviour. Intact expression is needed in motor neurons or/and *FoxP-iB* positive neurons. Our results hint to a potential *aPKC-FoxP* interaction. Overexpression of *aPKC* in adult flies led to improved learning ability. No overlap of *aPKC* and *FoxP* could be found in the brain, only in the VCN.

Zusammenfassung

Sprache ist ein zentraler Aspekt, der Meschen von anderen Tieren abgrenzt. *FoxP2* wurde als ein Gen identifiziert, das mit Sprache assoziiert ist. Das operante Sprachlernen zeigt Gemeinsamkeiten mit dem Drehmomentlernen von *Drosophila* im Flugsimulator: ein zunächst variables Verhalten wird durch eine operante Rückkopplung präzisiert. Fliegen mit einer *FoxP* Mutation zeigen Einschränkungen in ihrem operanten Selbst-lernen. Bei dieser Lernform haben die Fliegen, neben dem eigenen Verhalten, keine äußeren Anhaltspunkte zu den Parametern des Experiments. Um mehr über die zu Grunde liegenden Mechanismen zu erfahren, wurden *FoxP* auf verschiedene Weise manipuliert.

Es konnte gezeigt werden das *FoxP* in der adulten Fliege nicht für operantes selbst-lernen benötigt wird. Es ist jedoch wichtig für den Erhalt der Lernfähigkeit. Es konnte keine einzelne Gehirnregion gefunden werden, wo korrekte *FoxP* Expression für diese Lernverhalten nötig war. *PKC53e* ist ebenfalls für operanten Selbstlernen nicht erforderlich. Im Gegensatz dazu ist *aPKC* wichtig, die Korrekt Geneexpression wird in motorischen Neuronen sowie/oder in Neuronen die *FoxP-iB* exprimieren benötigt. Die Ergebnisse deuten auf eine Interaktion von *aPKC* und *FoxP* hin. Eine Überexpression von *aPKC* verbesserte das Lernvermögen der Fliegen. Es wurde keine Überlappung von *FoxP* und *aPKC* im Gehirn gefunden, jedoch im VNC

1. Introduction

1.1 Learning mechanisms and the "*Drosophila* flight simulator" (DFS)

When making learning experiments two main types can be distinguished. There is classical and operant conditioning. In classical conditioning the animal associates two external stimuli. While for operant conditioning a link between a behaviour and the respective outcome is formed, e.g. by pressing a lever to receive a reward. Classical or Pavlovian conditioning was developed by Ivan Pavlov in 1927 (Pavlov, 1927). He established one of the fundamental concepts of learning experiments, by limiting the behaviour of the test subject to study behaviour. In this case, he looked at the salvatory glands of dogs and put them therefore in a harness, limiting their movement. He presented food to the animal to cause salivation. He then noticed that the dogs were already starting to salivate, after they heard a bell sound indicating the experimenter with the food was about to enter. The unconditioned stimulus (US), in this case the food, was substituted by the previously neutral conditioned stimulus (CS), in this case the bell. After training presentation of the CS is sufficient to elicit a conditioned response (CR). It is necessary for this new association, that the CS can serve as a predictor of the US. For operant conditioning, first developed by Skinner, the animal learns about the outcome of its own behaviour (Skinner, 1935). By performing a specific action, e.g. pressing a lever, the animal is either rewarded or punished. It therefore links its behaviour to the stimulus. *Drosophila* has been a useful model organism to study behaviour (Alekseyenko et al., 2019; Balleine, 2019; Davis and Zhong, 2017; Oram and Card, 2022). In particular studies on learning behaviour gave valuable insight (Adel and Griffith, 2021; Colomb and Brembs, 2016; Georganta et al., 2021; Heisenberg, 2015). It was shown that operant learning can be split into two different forms of learning: a world-learning and self-learning component (Brembs, 2009; Brembs and Heisenberg, 2000; Brembs and Plendl, 2008; Wolf and Heisenberg, 1991). Both rely on different mechanisms (Brembs, 2011; Colomb and Brembs, 2010). For self-learning no external cues are provided to the animal. It can only predict the outcome based on its own behaviour. For the world-learning component, the fly is exposed to an external stimulus, e.g. colours, predicting for example a punishing event.

In a composite learning task, both components – self- and world-learning, are induced. In addition to punishment for flight attempts to the left or right (self-learning), the colour of the arena (world-learning) changes according from e.g. green for left to blue for right turning. In a normal training situation of 8 minutes the animal only learns the colours. When testing without the world-learning component the animals show no preference for either side. The world-learning is inhibiting the self-learning. Only with extended training time the animal can overcome this inhibition. By that, a transition of goal-directed behaviour to habit formation takes place. The mushroom bodys (MB) was identified as a potential site of interaction between both systems (Brembs, 2009). It is noteworthy that *FoxP* is not expressed in this brain area (Palazzo et al., 2020). While self-learning is independent on the cAMP pathway in *Drosophila*, manipulations of *PKC* abolish this type of learning (Brembs and Plendl, 2008). On the other hand, world-learning requires cAMP but is independent of PKC. This mechanism can not only be seen in *Drosophila* but also, for example, in the sea slug *Aplysia*. Also here cAMP pathways are not required for self-learning, but PKC is necessary (Lorenzetti et al., 2008).

Using the "*Drosophila* flight simulator" (DFS) it was possible to illustrate, that operant conditioning is more complicated than it seems. The DFS was first developed by Götz (1964). It is a very versatile set-up, enabling the performance of many different types of experiments. A fly is attached to a torquemeter and is flying stationary in an arena. The arena is homogeneously illuminated. Depended on the research question it is possible to add colours or patterns during the experiment. An infrared laser is used as a negative stimulus. Although *Drosophila* is not a vocal species, the torque learning in the DFS is showing strong similarities to the basic mechanism of song learning in birds. A juvenile finch first produces a very variable sub-song, trying to imitate the correct vocalisation of the parents. This sub-song is changed more and more to match the correct output, with an operant feedback loop. The expected outcome (correct adult song) is continuously compared to the actual vocalisation and then changed accordingly (Bolhuis et al., 2010; Brainard and Doupe, 2013; Day et al., 2019). In the DFS, the fly is also producing a very variable behaviour, using the whole range of motion. It is than trained to prefer one turning direction over the other, using an infrared laser as negative stimulus. This is coupled either to the left or right side.

The animal is now, like in the previous example, changing the initially variable behaviour to a narrower range to the unpunished side. The desired outcome (no heat) is compared to the current state (heat) and then changed with a similar operant feedback loop (Mendoza et al., 2014; Mooney, 2004). It has been shown that *FoxP* mutant *Drosophila* are not able to perform this self-learning task when the animal has only its own behaviour as a predictor of outcome. But, they are still able to perform a world-learning task. (Mendoza et al., 2014). This again points towards the similarity of speech learning in e.g. birds and the torque learning for flies.

1.2 The *FoxP* gene

The *Forkhead box P* (*FoxP*) gene encodes a highly conserved transcription factor. It consists of the name giving forkhead box domain, a leucin zipper domain and an unstructured domain. Even though forkhead is a binding domain, the main binding affinity of the protein is controlled by the leucin zipper and the unstructured domain (Thulo et al., 2021). In general, *FoxP* has been reported to repress gene expression (Li et al., 2004; Spiteri et al., 2007; Vernes et al., 2007).

FoxP is present in a wide variety of species. Studies were performed in humans, rats, songbirds as well as in flies. (Chen et al., 2013; Gaub et al., 2016; Groszer et al., 2008; Norton et al., 2019; Teramitsu, 2004). The highly conserved sequence and structure indicate evolutionary selection (Enard et al., 2002; Haesler, 2004). As the conserved structure indicates, *FoxP* also serves a similar function across species. Defects in this gene have been shown to cause defects in vocalisation or speech learning in humans and birds (Chen et al., 2013; Groszer et al., 2008; Haesler et al., 2007; Lai et al., 2001).

In *Drosophila*, it was shown that besides learning *dFoxP* is also important in a variety of different behaviours like courtship and motor coordination (Lawton et al., 2014). Also, the ability and speed of decision making seems to be affected (DasGupta et al., 2014). Differences could also be observed in the turning behaviour (Kottler et al., 2019). Several temporal or spatial patterns were impaired when *FoxP* was manipulated (Palazzo et al., 2020)

Due to this important and unique role for language in humans *FoxP* is an important study subject that could help to unravel some basic mechanisms. In humans, four paralogs of *FoxP* can be found, *FoxP1-4*. *Drosophila* has only one gene, but it has three different isoforms (Castells-Nobau et al., 2019; Palazzo et al., 2020; Santos et al., 2011). The *Drosophila FoxP-iB* isoform seem to be the most relevant one and will be a focus in this study (Palazzo et al., 2020).

Usually, genes that are relevant for studies in humans are first discovered in model organisms. The homologs in humas that would be relevant for the study of diseases are then discovered later. Interestingly, it was the other way around for the discovery of the *FoxP* gene. It was first discovered in humans, later in other organisms. It was also the first gene associated with speech learning and development (Lai et al., 2001). Language is a key characteristic of humans, distinguishing them from other animals. Studying a gene that is impacting this fundamental function should yield important insight about the underlying mechanism. *FoxP* is studied in a variety of different contexts. It is analysed for developmental effects (Castells-Nobau et al., 2019; Palazzo et al., 2020), disease models (Co et al., 2020), evolution (Villalobos et al., 2021; Zhang et al., 2002), and learning (Chen et al., 2013; Mendoza et al., 2014).

Figure 1: Expression pattern of FoxP in the Drosophila brain

In *Drosophila*, *dFoxP* was first discovered in 1987 (Weigel et al., 1989). It is expressed in a wide variety of regions in the *Drosophila* brain and is important for development (Castells-Nobau et al., 2019). It is expressed e.g. in the protocerebral bridge (PCB), the fan shaped body (FS) and the noduli, that are part of the central complex. This highly interconnected area processes environmental information and controls motor outputs. It is therefore essential for behaviour (Pfeiffer and Homberg, 2014; Wolff and Rubin, 2018). Further, *FoxP* expression can be found in the saddle, corresponding to the antennal mechanosensory and motor center (AMMC) (Chiang et al., 2011). This area receives input from antenna neurons. The ellipsoid body (EB), also part of the central complex, dose not express *FoxP*. Also, no expression can be found in the mushroom body (MB) (Palazzo et al., 2020) (Fig. 1). The MB is described as an important brain area for associative learning, but mostly in the context of olfactory learning (Adel and Griffith, 2021; Davis, 2005, p. 20; Heisenberg, 2003). *FoxP* is expressed in the ventral nerve cord (VCN) and in motor neurons as well (Palazzo et al., 2020).

The tools to analyse the behavioural effects of *FoxP* in *Drosophila* were lacking. Due to the availability of new *FoxP* lines (*g-RNA* and *Gal4/LexA*) we are trying to get deeper insights in the role of *dFoxP* for learning in *Drosophila*.

1.3 Protein kinase C (PKC) gene family

Protein kinases are defined as enzymes that phosphorylate proteins (Hunter, 1991). The Protein kinase C (PKC) gene family consists of highly conserved serine/threonine kinases. They share carboxy-terminal kinase domain together with an amino-terminal regulatory domain (Rosse et al., 2010). The inactive PKC is autoinhibited by a pseudosubstrate domain in the regulatory domain that blocks substrate interactions (Pears et al., 1990). For the activation a second messenger is needed, diacylglycerol (DAG), lipid or Ca^{2+}. Binding to the regulatory domain displaces the pseudosubstrate from the catalytic site (Nalefski and Newton, 2001). In *Drosophila* there are five genes. Two classical PKCs, *protein C kinase 53E* (*Pkc53E*) and *inactivation no afterpotential C* (*inaC*), tow novel PKCs, *protein C kinase 98E* (*Pkc98E*) and *protein kinase C δ* (*Pkcδ*) and one *atypical PKC* (*aPKC*).

Classical PKCs are activated by DAG and Ca^{2+}, novel PKCs only need DAG and aPKC is independent of both (Mukai, 2003; Shieh et al., 2002).

PKCs are often studied in the context of cell polarity and development or tumour regulation (Archibald et al., 2015; Broughton et al., 1996; Manning et al., 2002; Rosse et al., 2010; Shieh et al., 2002; Sopko et al., 2014). In addition, it was reported that they are important for learning or memory maintenance in snails (Bougie et al., 2012, 2009; Cai et al., 2011; Chesnokova et al., 2019; Lorenzetti et al., 2008), flies (Colomb and Brembs, 2016) and birds (Sakaguchi and Yamaguchi, 1997; Yoshida et al., 2003)

1.4 Drosophila melanogaster genetic toolbox

With the popularity of *Drosophila*, a wide variety of different genetic tools was developed. A prominent one is the UAS/Gal4 system. It consists of an upstream activation sequence (UAS) effector line and a Gal4 driver line. For the activation of the UAS sequence Gal4 has to bind to it, expressing a sequence under UAS control. It is therefore possible to limit the effects locally, depending on the Gal4 expression pattern (Brand and Perrimon, 1993). A wide range of Gal4 lines are available for *Drosophila*. On the website of the “Bloomington Drosophila Stock Center”, a prominent place for ordering fly strains, 16243 entries can be found, when searching for Gal4 (https://bdsc.indiana.edu, 29.09.22). When combing this system with Gal80 or GSGal4 a temporal component can be introduced. The Gal80 is temperature sensitive and represses the Gal4 expression at 18°C. By transferring it to 30°C it gets inactivated, enabling Gal4 activation. The GSGal4 system only expresses Gal4 when animals are fed with the steroid hormone RU486.

Another important technique is the CRISPR/Cas9 system. Adapted from the bacterial immune system it allows introduction of targeted gene mutations (Bassett and Liu, 2014). The CRISPR sequence (clustered repetitive interspaced short palindromic repeats) consist of about 20 nucleotides. This guide RNA (gRNA) provides a template for the Cas9 protein that will cut the according sequence out of the DNA. This enables targeted knockout of genes. In combination with the UAS/Gal4 system the versatility of this system is even further increased.

There are several ways of silencing neurons in *Drosophila*. Two prominent ones are the Tetanus toxin light chain (TeTx) or human inward rectifying potassium channel (Kir2.1). Former is cleaving neuronal synaptobrevin, that is essential for neurotransmitter release (Sweeney et al., 1995). Kir on the other hand is silencing the neurons by hyperpolarizing the cells. This leads to blocking of action potentials (Nitabach et al., 2002). Both methods are leading to the same effect: silencing targeted neurons.

1.5 Aim of the study

The goal of this study was to disentangle the involvement of *FoxP* in operant self-learning. It was shown that *FoxP* mutants are impaired in their self-leaning ability. It was unknown in what brain areas the expression is needed or at what time it is necessary.

Newly created *FoxP* lines in combination with the use of the DFS setup provided a combination to investigate this question to get future insights. Utilizing a self-learning paradigm, the effect of different *FoxP* spatial or temporal manipulation are tested.

The block of certain brain areas supposedly inhibited operant self-learning. It was attempted to reproduce this findings.

PKC was also shown to be involved in operant self-learning. Since it is involved in this learning type, like *FoxP*, a possible interaction of *FoxP* and *PKC* was investigated.

2. Material and methods

2.1 Fly care

If not stated otherwise flies were raised on standard cornmeal/molasses medium at 25°C and 60% humidity at a 12-hour light/dark cycle. For experiments requiring the expression of temperature sensitive Gal80 system animals were raised at 18°C. For behavioural experiments 20 females were placed together with five to eight males and were allowed to lay eggs for 24 hours. They were flipped daily into fresh vials, ensuring the same larval density. Flies were prepared the day before the experiment, allowing them time to recover. 24 to 48 hours old female flies were briefly immobilized using cold anaesthesia. A thin triangular copper hook (0,05 mm diameter) was glued (3m espe sinfony, 3M Deutschland GmbH) between head and thorax, fixing both body parts to each other. Each animal was kept individually in a small moist chamber with a few grains of sugar. For the *UAS-PKCi* experiments flies received a heat-shock at 35°C for four hours before the test. For *tub-Gal80* expression animals were placed at 30°C for two days. Experiments were always performed at 25°C. For experiments that were utilizing the gene-switch system newly hatched flies were placed on instant food containing the steroid hormone RU486 (200 µg/ml) for two days.

2.2 Fly strains

Table 1: Table of fly strains

genotype	use	Bloomington	Flybase
;;ato-Gal4	driver line		
C380-Gal4;;	driver line	80580	FBti0016294
;;D42-Gal4;	driver line	8816	FBti0002759
;;FoxP-iB-Gal4/TM3	driver line		
;;FoxP-LexA;	driver line		
;;GMR11F02-GAL4	driver line	49828	FBti0132980
;;GMR20A02-GAL4	driver line	48870	FBti0133737
;;GMR20H05-GAL4	driver line	47896	FBti0133817
;;GMR48A03-GAL4	driver line	50339	FBti0136204
;;GMR52B10-GAL4	driver line	38820	FBti0136576
;;GMR55G08-GAL4	driver line	50422	FBti0136906
;;GMR64H04-GAL4	driver line	39323	FBti0137498
;;GMR65A06-GAL4	driver line	39330	FBti0137511
;;nSyb-GS	driver line	80699	FBti0201287
ELAV-Gal4;;	driver line		
ELAV-Gal4;Tub-Gal80ts;;	driver line		
nSyb-GAL4	driver line		
y[1] w[*]; Mi{Trojan GAL4.un} aPKC[MI10848-TG4.un]/SM6a	driver line	77814	FBti0196316
;;g-aPKC	effector line	85862	FBti0210993
;;g-BAZ	effector line	84234	FBti0207133
;;g-PKC53e	effector line	76612	FBti0194968
;;UAS-aPKCdelta	effector line	51673	FBti0154819
;;UAS-Kir2.1	effector line	6596	FBti0017551
;;UAS-PKCi	effector line	4589	FBti0010565
;;UAS-t:gRNA(4xFoxP)	effector line		
;LexAop-mCD8::RFP/UAS-mCD8::GFP;;	effector line		
;UAS-Cas9;;	effector line		
;;UAS-Cas9;	effector line		
;UAS-CD8::GFP;;	effector line		
;UAS-TeTxG	effector line	28838	FBti0038527
;UAS-TetxE	effector line	28837	FBti0038528
Canton S (CS-TZ)	wild type strain		
Wild type Berlin	wild type strain		

2.3 Experimental Set-up

Two different set-ups were utilized for the experiments. First the “Shiming-set-up” was used (Tang and Juusola, 2010). After the core device was damaged and unable to work reliably anymore the work was continued with the “Götz-set-up” (described in Götz 1964). Prepared flies (see above) were attached via a clamp to the torquemeter. The device measures the rotational force (torque) around a horizontal axis. The animal is placed into a cylindric panorama (arena diameter 58 mm), that is homogenously illuminated from behind by a projector (Götz: DLPLCR4500EVM, Texas Instruments) or a halogen lamp (Shiming: OSRAM 100W/12V). With this set-up stationary flight in a controlled environment flight was achieve. An infrared laser (StockerYale Lasiris SNF series; 825 nm, 150 mW) was used to punish the flies. It was pointed from above onto the animal’s head. The laser was pulsed (approx. 200ms pulse width ~4 Hz) and the intensity was adjusted. The experiment is fully computer controlled, using a custom program (LabView, National Instruments) (RRID:SCR_014325).

For the “Shiming device” the arena rotation for the optomotor stimulus was switched on by hand. The rotation was reversed after the fly had reach its opto-motor (om) peak. Unlike the “Götz device”, where the rotation is automatically controlled by the software, the om periods are not recorded and were not analysed. For all „Shiming“ experiments the periods are numbered from 1 to 9 (Table 2). For the “Götz device” the first and last four periods are om-periods. The full experiment consists of 17 periods (Table 3). Since no PI can be calculated for om periods, these are omitted in the PI plots. Therefore periods 5 to 13 are plotted.

2.4 Experimental design

For all behavioural experiments a self-learning paradigm was chosen. The animal had only its own behaviour to deduce the outcome, no shapes or colours were provided as additional information. At the beginning of each experiment the om-response of the fly was recorded for two minutes with four opto-motor periods (30 seconds each). A rotating stripe pattern is presented going clockwise or counter-clockwise, alternating between the periods.

For the Shiming-setup this was done manually. As the fly tries to stabilize the stripes, it produces torque to the corresponding direction (Bausenwein et al., 1986).

The trace was adjusted to achieve an equal magnitude of left and right torque signal. 0 should be therefore roughly equal to flying straight. The main experiment consisted of nine periods of two minutes (if not stated otherwise). The laser was off for the first two periods, so that the fly could freely choose its direction of flight. In the following two training periods either the left or the right torque was coupled with the punishing stimulus. It was alternated between experiments. The training periods were followed by one test period without punishment. Afterwards the fly was trained again with the same side punished as before for two periods. Finally, no heat was applied in the final two test periods. The experiment was completed by further four 30 seconds opto-motor periods. As a quality control the fly was exposed to the laser after the experiment, to ensure it was correctly adjusted. If the fly survived for 15 seconds it was discarded. In addition, flies that did not show any or a shifted OM trace, indicating an error with the measuring device, were excluded. Based on such OM trace drift, a damage of the Shiming device could be detected. Animals were also excluded if they had a strong positive preference and therefore were not trained properly. Lastly flies with poor flight performance (constant stopping of flight) were also excluded.

Table 2: Experimental design "Shiming Setup"

Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Period 7	Period 8	Period 9
Pretest	Pretest	Training	Training	Test	Training	Training	Test	Test
No heat	No heat	Heat	Heat	No heat	Heat	Heat	No heat	No heat

Table 3: Experimental design "Götz Setup"

OM bevore				Experiment									OM after			
Period 1	Period 2	Period 3	Period 4	Period 5	Period 6	Period 7	Period 8	Period 9	Period 10	Period 11	Period 12	Period 13	Period 14	Period 15	Period 16	Period 17
OM	OM	OM	OM	Pretest	Pretest	Training	Training	Test	Training	Training	Test	Test	OM	OM	OM	OM
No heat	No heat	No heat	No heat	No heat	No heat	Heat	Heat	No heat	Heat	Heat	No heat	No heat	No heat	No heat	No heat	No heat

2.5 Statistical analysis

The preference of a fly for right or left torque was quantified as the performance index PI:

$$PI = (a - b) / (a + b)$$

a is referring to the time the animal spent on the unpunished site during training. b is referring to the time spent on the site that is punished. A PI of 1 would therefore indicate that the animal spent 100% of the time on the unpunished side. A PI of -1 would indicate that the fly only spent its time on the punished side. All data is analysed using R (R Project for Statistical Computing) (RRID:SCR_001905). The evaluation script can be found in the github repository (https://github.com/brembslab). The first test period after the fourth training period was plotted. A P-value of 0.005 was used for significant difference level.

2.6 Image acquisition

One to three days old flies were fixated in 4% PFA solution for 2 hours at 4°C. The dissected brains were then mounted on object slides and sealed with Vectashield (Vector Laboratories, Burlingame, CA). Scans were performed using a Leica SP8 confocal microscope (RRID: SCR_018169) with 20x immersion oil objective. Image stacks were analysed using ImageJ (Version 1.53k, RRID: SCR_003070). The contrast and brightness were only generally adjusted.

2.7 Data availability

All raw data can be accessed at:

https://epub.uni-regensburg.de/cgi/search/archive/advanced?screen=Search&dataset=archive&_action_search=Suchen&documents_merge=ALL&documents=&title_merge=ALL&title=&creators_name_merge=ALL&creators_name=Ehweiner&creators_id_merge=ALL&creators_id=&creators_orcid=&editors_name_merge=ALL&editors_name=&editors_id_merge=ALL&editors_id=&editors_orcid=&date=&id_number_name_merge=ALL&id_number_name=&abstract_merge=ALL&abstract=&keywords_merge=ALL&keywords=&publication_merge=ALL&publication=&publisher_merge=ALL&publisher=&book_title_merge=ALL&book_title=&series_rgbg_merge=ALL&series_rgbg=&series_merge=ALL&series=&teaching_series_merge=ALL&teaching_series=&subjects_merge=ANY&institutions_merge=ANY&projects_merge=ALL&projects=&network_merge=ANY&research_group_merge=ANY&department_merge=ALL&department=&referee_merge=ALL&referee=&isbn_merge=ALL&isbn=&classification_name_merge=ALL&classification_name=&own_doi_merge=ALL&own_doi=&ranking_merge=ANY&own_properties_merge=ALL&own_properties=&satisfyall=ALL&order=-date%2Fcreators_name%2Ftitle

For links to individual data sets see supplement.

3. Results

3.1 Temporal *FoxP* knock-out

3.1.1 Immediate effect of *FoxP*-loss

The newly created guide RNA (gRNA) line for the *FoxP* gene enables a conditional knockout under temporal and/or spatial control. Knockout in neurons during the embryonal stage led to strong motoric effects with impairment in walking. Since the flies were also unable to fly, they could not be tested in the flight simulator. The knockout was therefore limited to adult flies. Three groups were tested in parallel (Fig. 2). The experimental group expressed the Cas9 protein and the gRNA together enabling *FoxP* knockout. The two controls expressed either the Cas9 protein or the *FoxP* gRNA (*gFoxP*) only, not affecting *FoxP* expression. The experiment was performed two times, using different genetic tools for the temporal control and two different set-ups. First, the temporal knockout was achieved using the Gal4 repressor *tub-Gal80*. After being raised at 18°C the flies were transferred to 30°C for two days before the experiment. Due to issues concerning the fly crosses and the “Shiming device” the experiment was terminated at an early stage. The experimental group showed a clear ability to learn and was significantly different from 0 ($p = 0.00164$). Contrary to the expectation, the *gFoxP* control showed an increase in the PI in the first test after training but was not significant ($p = 0.0147$). The *Cas9* control were not different from 0 ($p = 0.4$), but the desired sample size of 20 was not reached (Fig. 2, experimental sequence Fig. S1). As control flies do not exhibit any genetic manipulation it was assumed that these should show significant learning behaviour.

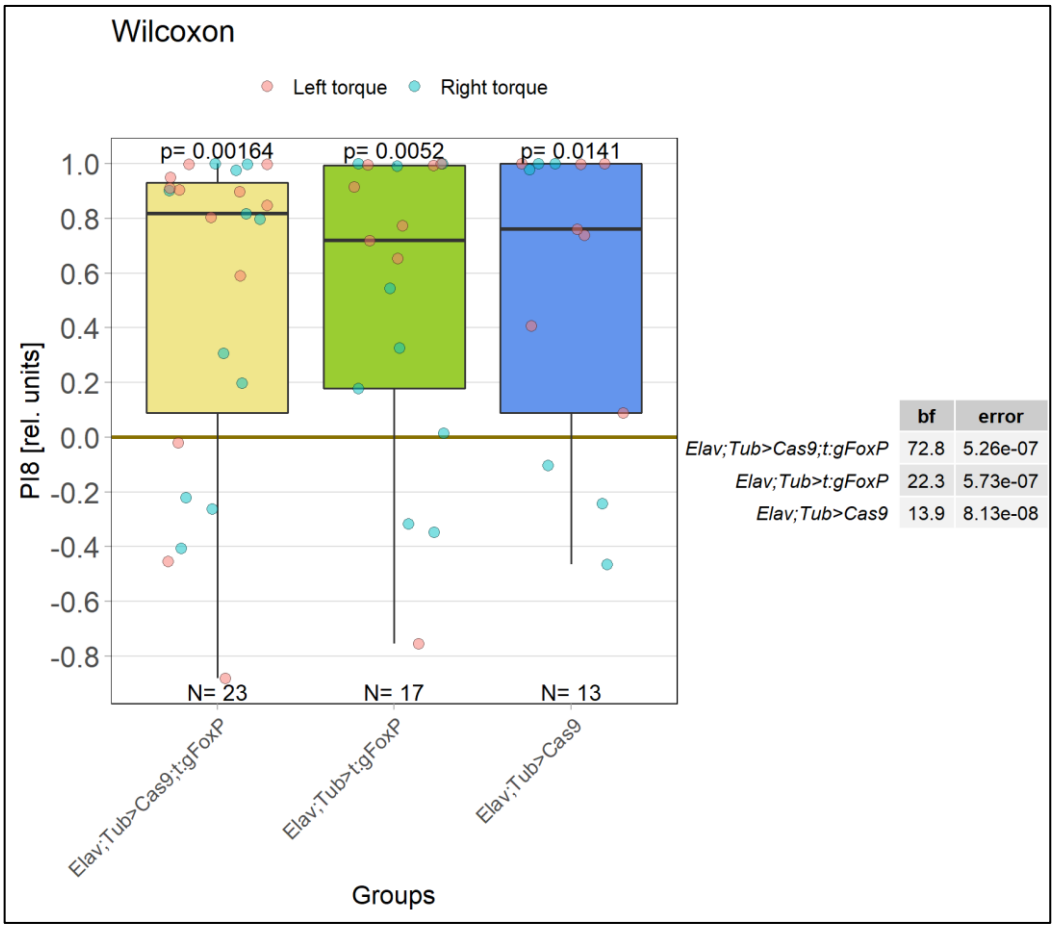

Figure 2: Conditional FoxP knockout in adult flies. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 8, x-axis: tested groups: Elav-Gal4;tub-Gal80>UAS-Cas9;UAS-t:gFoxP, Elav-Gal4;tub-Gal80>UAS-t:gFoxP, Elav-Gal4;tub-Gal80>UAS-Cas9. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

For reproduction of the experiment a different set-up (“Götz-device”) and a different genetic tool (gene-switch) were used to validate the previous results. Newly hatched flies were transferred to vials containing the steroid hormone RU486 for two days before the experiment. This time both controls showed an increased PI during testing and are significantly different from 0 ($p = 0.0000105$ and $p = 0.000483$ respectively). The experimental cross with knocked out *FoxP*, was also significantly different from 0 after training ($p = 0.000235$) (Fig. 3, experimental sequence Fig. S2).

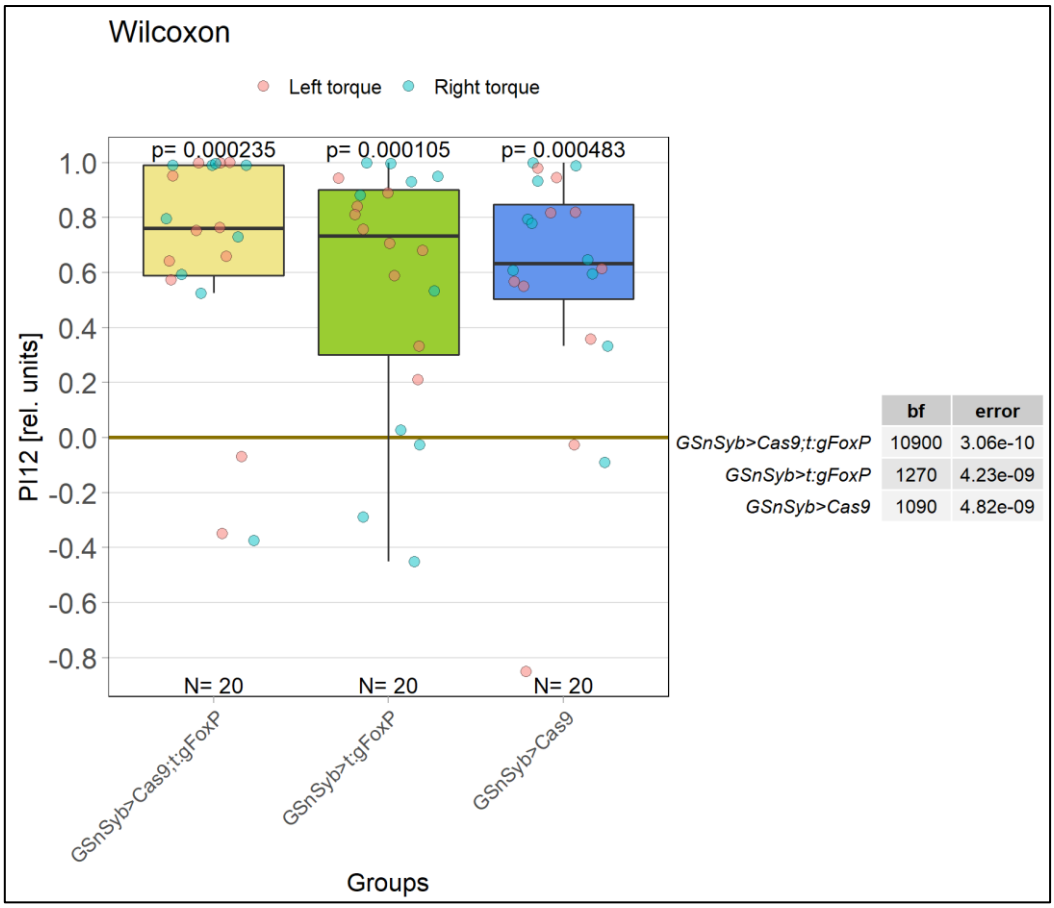

Figure 3: Conditional FoxP knockout in adult flies. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: nSyb-GS>UAS-Cas9;UAS-t:gFoxP, nSyb-GS>UAS-t:gFoxP, nSyb-GS>UAS-Cas9. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

Both experiments showed the same results (Fig. 2, Fig. 3). Even if *FoxP* was not expressed in adult flies, they were still able to perform the self-learning task. A potential role of *FoxP* for operant self-learning could not be proved.

3.1.2 Aging effect of *FoxP*-loss

The *FoxP* knockout showed no immediate negative effect on learning behaviour. The question remained if the absence would lead to any long-term effect. Since *FoxP* is a transcription factor a delayed effect could not be excluded. Therefore, the previous temporal knockout, utilising the gene switch system, was tested again. After being placed on the RU486 for 48 hours, flies were kept in vials for 12 days. Additionally, flies were kept without RU486 as internal control.

14-day old flies were then tested for operant self-learning (Fig. 4, experimental sequence Fig. S3). Since there was no difference between the *Cas9* or gRNA control groups with and without RU486, they were all pooled together (t:gFoxP/Cas9). The effector control flies were still able to learn and were significantly different from 0 (p = 0.000187). The control flies not exposed to RU486 also showed an increased PI after training and were significantly different from 0 (p = 0.00219). The experimental cross exposed to RU486 showed a clear learning impairment. The PI was not increased after training and was not different from 0 (p = 0.121). Thus, *FoxP* expression was necessary for the maintenance of the learning ability of aging flies. While animals with intact *FoxP* expression still performed the task after 14 days, a loss of *FoxP* led to learning impairment.

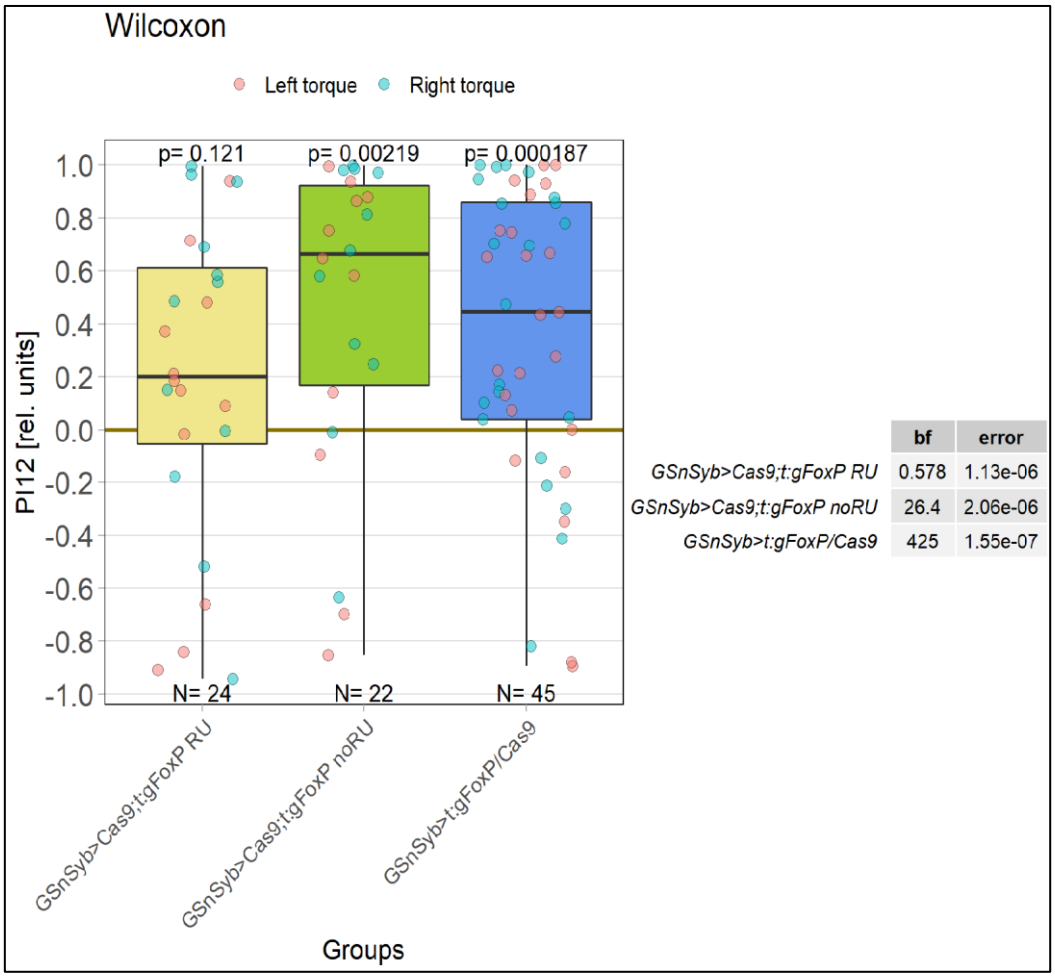

Figure 4: Testing of 14-day old flies with adult FoxP knockout. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: nSyb-GS>UAS-Cas9;UAS-t:gFoxP with RU, nSyb-GS>UAS-Cas9;UAS-t:gFoxP without RU, nSyb-GS>UAS-t:gFoxP or nSyb-GS>UAS-Cas9. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

To investigate at which time point an impairment in learning ability can be detected flies were tested seven days post treatment. Since the effector control flies were still able to learn after 14 days, they were not tested again. It was assumed they would also be able to learn after seven days since learning performance tend to decrease over time (Brenman-Suttner et al., 2020; Guo et al., 1996). Both the experimental cross and the genetic control were significantly different from 0 (p= 0.000607 and p = 0.000373). Flies without *FoxP* were still able to learn when tested after seven days (Fig. 5, experimental sequence Fig. S4). Therefore, it can be assumed that *FoxP* is involved in operant self-learning in age dependent manner. A decrease in learning performance could not be seen immediately or after seven days. After 14 days though, a clear impairment could be observed.

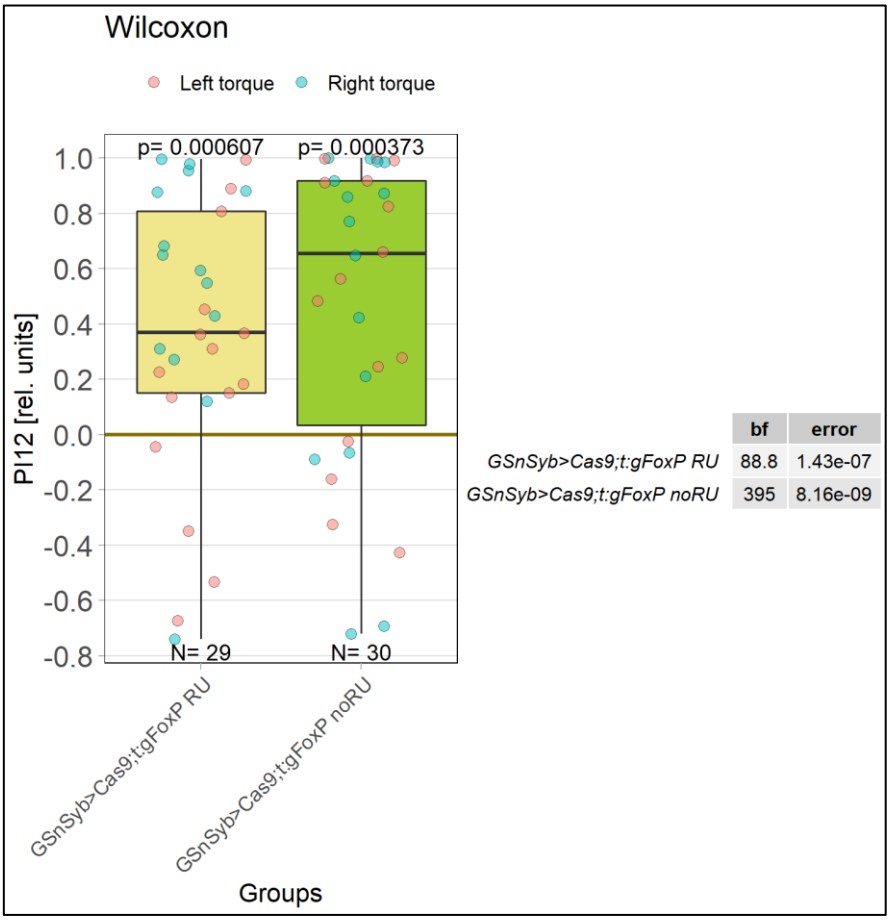

Figure 5: Testing of seven-day old flies after FoxP knockout. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: nSyb-GS>UAS-Cas9;UAS-t:gFoxP with RU or without RU. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

3.2 Local *FoxP* knockout

As panneuronal temporal knockout of *FoxP* did not result in immediate impairment. *FoxP* was knocked out via Cas9/gRNA constructs independent of the developmental stage. Brain regions with prominent *FoxP* expression were targeted. The experimental group expressed the Cas9 protein and the gRNA together enabling the knockout of *FoxP*. The two controls expressed either the Cas9 protein or the *FoxP* gRNA only, not affecting *FoxP* expression.

3.2.1 *FoxP* knockout in the central complex

3.2.1.1 Protocerebral bridge

Two different Gal4 lines with slightly different expression patterns in the protocerebral bridge (PCB) were tested. *GMR55G08-Gal4* was tested with the “Shiming-device” (Fig. 6). This line shows the main overlap in the PCB (Palazzo et al., 2020). The first control, only expressing the gRNA, showed an increased PI after training but was not significantly different from 0 ($p = 0.00619$). The *Cas9* control was not significantly different from 0 ($p = 0.125$) as well. Contrary to the usual case, flies showed increased learning performance during the second test after the last training (Fig. S5). This could indicate learning effects for the control group, since *FoxP* expression should have also not been altered. The experimental group showed an increased PI after training and was significantly different from 0 ($p = 0.00349$). As flies with knocked out *FoxP* in the PCB showed significant learning behaviour, it can be assumed that *FoxP* in this brain region does not interfere with operant self-learning.

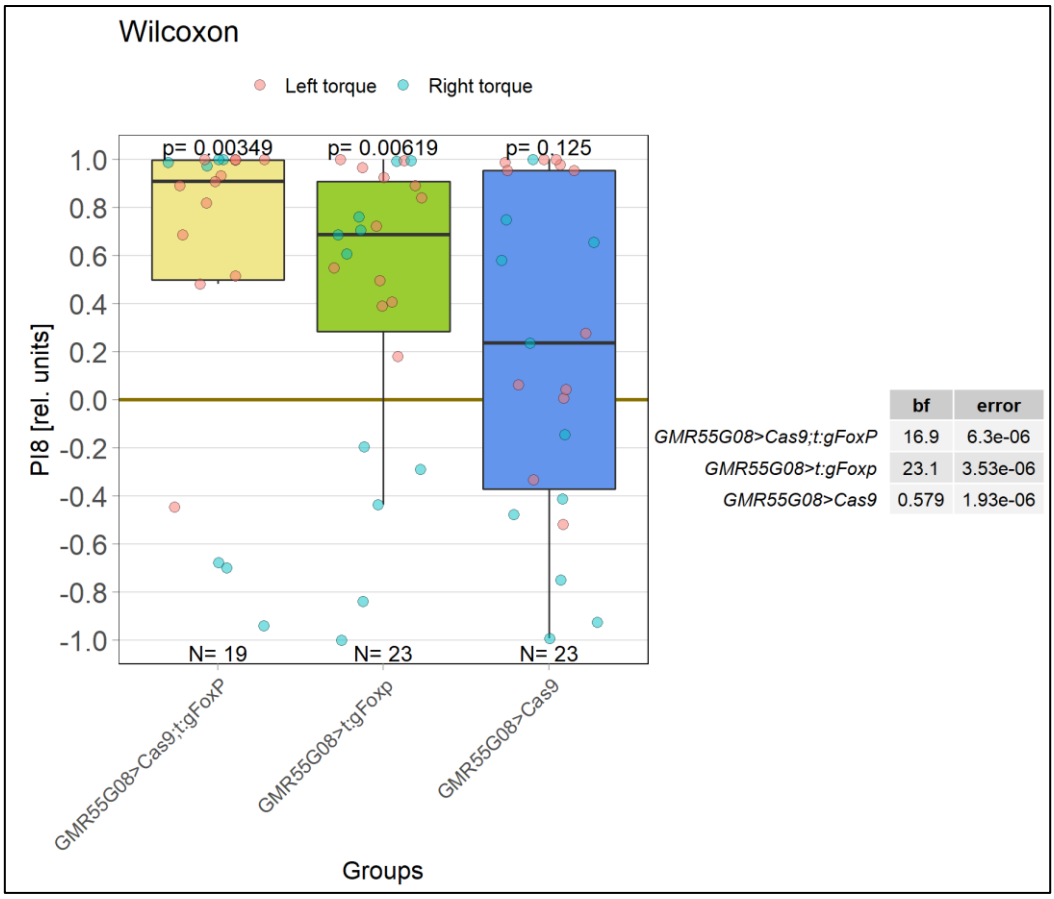

Figure 6: Local knockout of FoxP in the PCB. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 8, x-axis: tested groups: GMR55G08-Gal4>UAS-Cas9;UAS-t:gFoxP, GMR55G08-Gal4>UAS-t:gFoxP, GMR55G08-Gal4>UAS-Cas9. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

The *GMR65A06-Gal4* line was tested using the “Götz-device”. The overlap with *FoxP* can be seen in the top right panel (Fig. 7). The g*RNA* as well as the *Cas9* control group showed an increased PI and were both significantly different from 0 (p = 0.000134 and p = 5.25e-05 respectively). The experimental cross also showed a clear increase in the PI but was not significantly different from 0 in the first test period after the last training (p = 0.0076). However, here again an increased performance could be observed in the second test period after training (Fig. S6). As mentioned above, the PI tends to decrease in the second last test period. Therefore, it can be suggested that flies still can from memory after *FoxP* knock out in PCB.

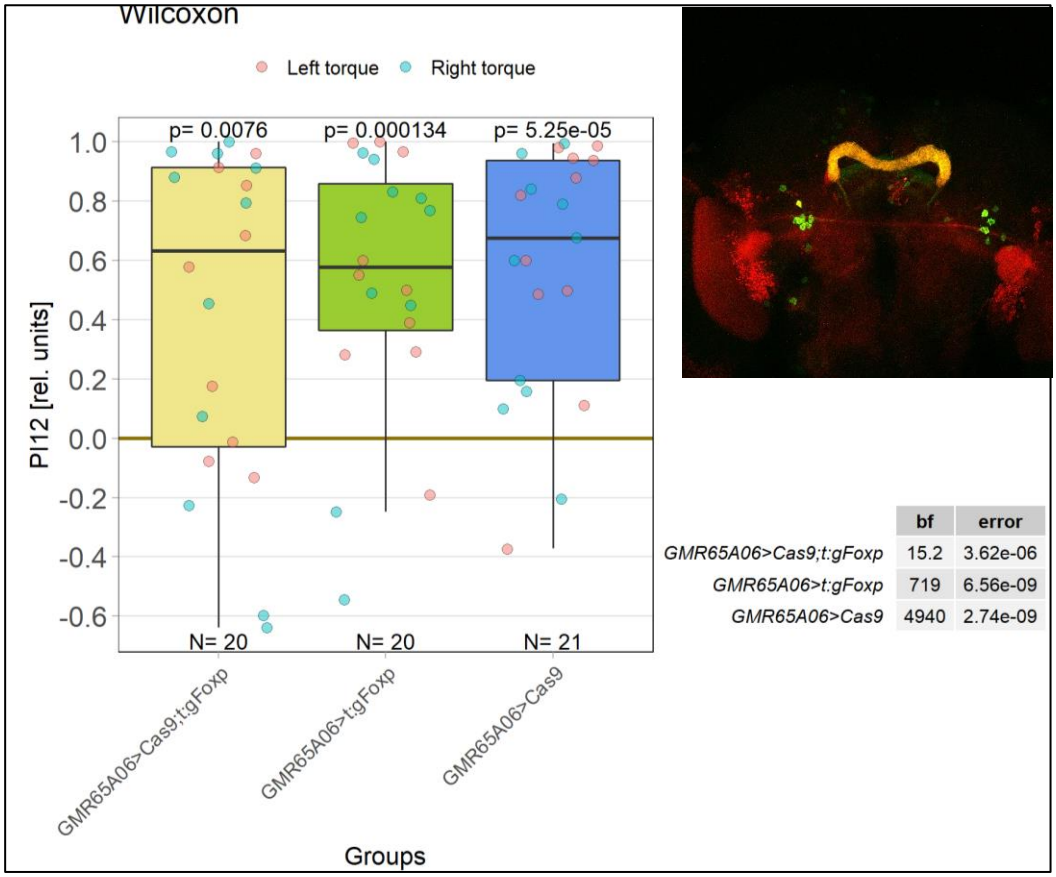

Figure 7: Local FoxP knockout in the PCB. Left panel: Performance index (PI) for the first period after the last training. Y-axis: PI of period 12, x-axis: tested groups: GMR65A06-Gal4>UAS-Cas9;UAS-t:gFoxP, GMR65A06-Gal4>UAS-t:gFoxP, GMR65A06-Gal4>UAS-Cas9. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics. Top right panel: coexpression of GMR65A06 (green) with FoxP (red), yellow shows overlap.

Both experiments seem to show the same result. Each time the animals were still able to learn, even though they were missing the normal *FoxP* expression mainly in the PCB (Fig. 6 and Fig.7). Spatial knock out of *FoxP* using both driver lines showed no impairment of learning behaviour.

3.2. Protocerebral bridge, fan-shaped body and noduli

GMR20H05-Gal4 line expresses in PCB, FB and noduli (Fig. 8). Both control groups showed learning behaviour as expected (*gFoxP* control: p = 0.0000708, *Cas9* control: p = 0.00271). The PI of the experimental cross was increased after training and was significantly different from 0 (p = 0.00365) (Fig. 8, experimental sequence Fig. S7). Experimental flies showed a poor flying performance and stopped regularly to fly.

Thus, *FoxP* expression in the targeted brain areas might not affect operant self-learning.

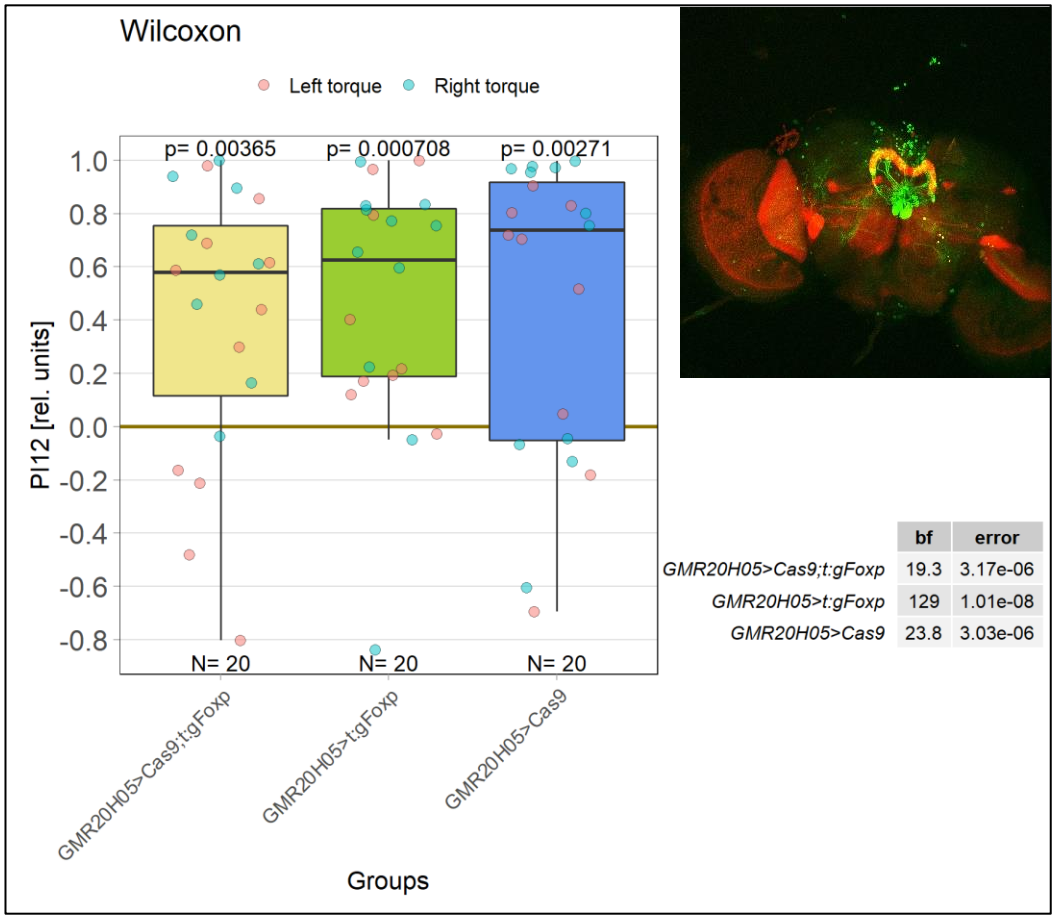

Figure 8: Local FoxP knockout in the PCB, FB and noduli. Left panel: Performance index (PI) for the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: GMR20H05-Gal4>UAS-Cas9;UAS-t:gFoxP, GMR20H05-Gal4>UAS-t:gFoxP, GMR20H05-Gal4>UAS-Cas9. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics. Right panel top: coexpression of GMR20H05 (green) with FoxP (red), yellow shows overlap.

3.2.2 Ato-Cluster

FoxP expression shows overlap in the ato-cluster (Palazzo et al. 2020). It was therefore targeted. The two control crosses showed an increased PIs after training and were both significantly different from 0 (p = 0.00137 and p = 0.0000447 respectively) (Fig. 9, experimental sequence Fig. S8). The experimental group also showed an increased PI in both test periods and was significantly different from 0 (p = 0.00166). Presumably, *FoxP* is not important in the ato-cluster for operant self-learning.

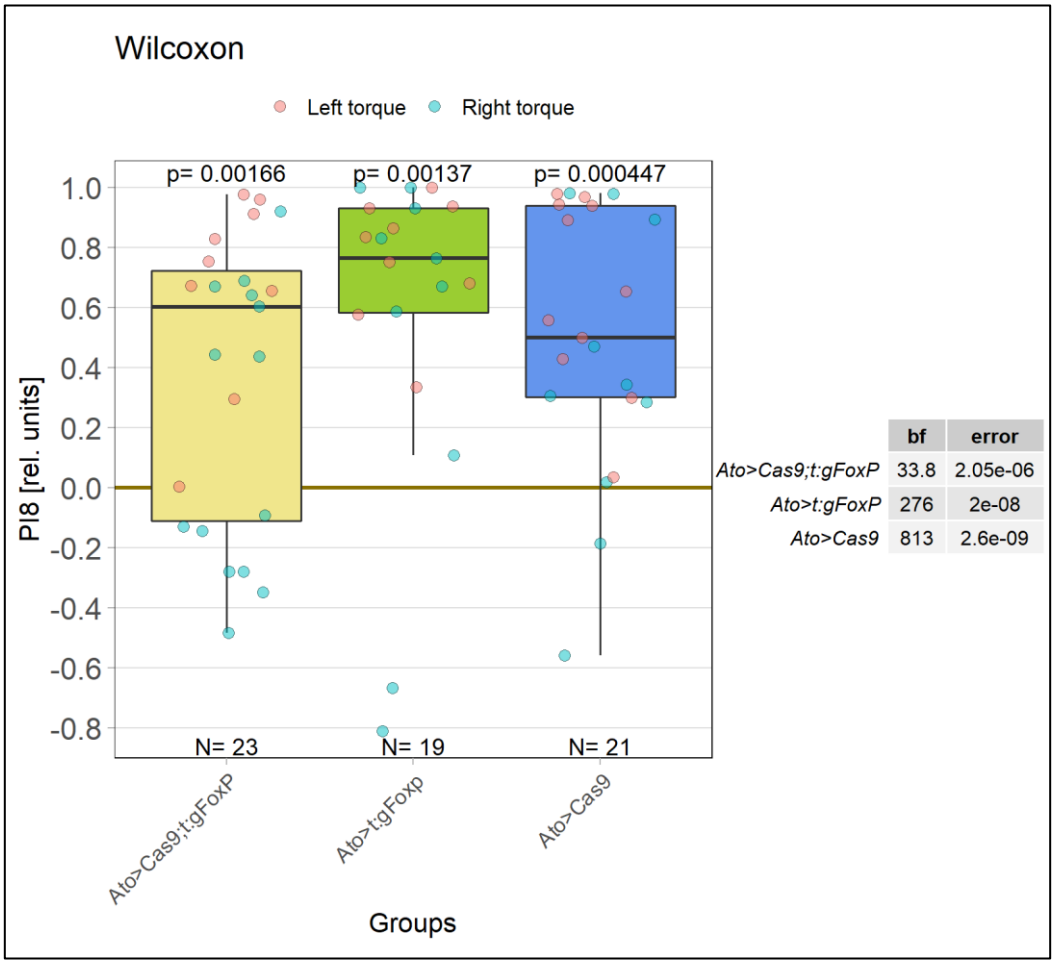

Figure 9: Local FoxP knockout in the Ato-cluster. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 8, x-axis: tested groups: Ato-Gal4>UAS-Cas9;UAS-t:gFoxP, Ato-Gal4>UAS-t:gFoxP, Ato-Gal4>UAS-Cas9. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

3.2.3 Missing areas for *FoxP* knockout

FoxP was knocked out in a Gal4 line targeting the saddle (Fig.10, experimental sequence Fig. S9). The *FoxP* control cross showed no learning effects (p = 0.0973), while the *Cas9* control showed an improved PI during testing (p = 0.000622). The learning performance of the experimental group in test period 12 was significantly different from 0 (p = 4.77e-05). However, following confocal scanning microscopy revealed no overlap of *Gal4* and *FoxP* expression (Fig. 11A). Since *FoxP* is not expressed in the targeted region, no effects on learning behaviour should be expected.

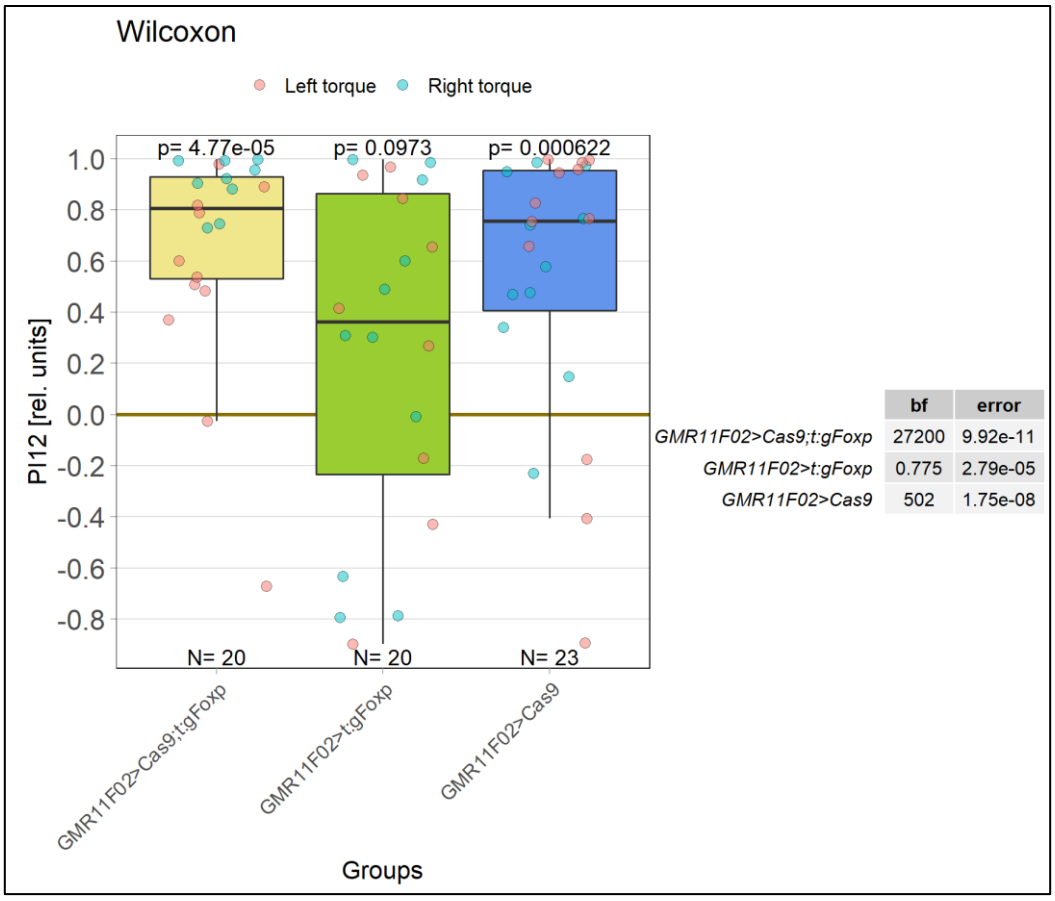

Figure 10: Local FoxP knockout in the expression area of GMR11F02 (no coexpression). Performance index (PI) for the first training period after the last training. Y-axis: PI of period 12, x-axis: tested groups: GMR11F02-Gal4>UAS-Cas9;UAS-t:gFoxP, GMR11F02-Gal4>UAS-t:gFoxP, GMR11F02-Gal4>UAS-Cas9. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

In addition, no overlapping line with expression in the vest was found. Line *GMR48A03* did not show any overlap with *FoxP* expression (Fig. 11B).

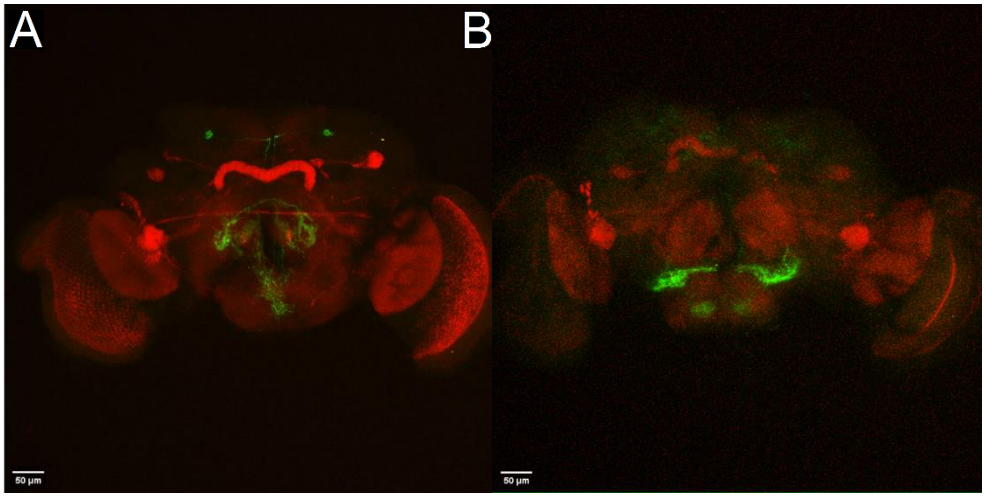

Figure 11: No overlap of FoxP and tested Gal4 lines. A: coexpression of FoxP-LexA (red) and GMR11F02-Gal4 (green. B: coexpression of FoxP (red) and GMR48A03-Gal4 (green)

The motor neurons were shown to be important for operant self-learning. But it was not possible to test the effect of *FoxP* knockout in motor neurons. Experiments with two different lines expressing in motor neurons were attempted, *D42-Gal4* and *C380-Gal4*. The experiment was stopped due to poor flight performance.

3.2.5 Local *FoxP* knockout summary

All experiments with local *FoxP* knockout showed similar results (Fig. 6-9). Manipulation of *FoxP* expression in neither of the target regions resulted in learning defects. The tested experimental crossings still showed learning behaviour. It was not possible to determine areas in the brain where *FoxP* expression could be necessary for operant self-learning in flies. However, not all areas with *FoxP* expression could be tested.

3.3 Local blocking of brain areas

No learning defects were observed when *FoxP* was knocked out in the protocerebral bridge (PCB). Blocking the whole region with TeTx did show learning defects according to the collaborating groupe of Liu (Fig 12A, B). In addition, blocking of the ellipsoid body (EB) also lead to learning defects (Fig 12C, D). No impairments were observed when the regions superior medial protocerebrum, saddle, vest or fan-shaped body were blocked (data not shown). To verify the results four Gal4 candidate lines were retested. Two of the lines expressed in the PCB (Fig.12A, B), the other two in the EB (Fig. 12C, D). In addition to using TeTx for blocking the neurons within the brain regions of interest we also decided to silence the respective neurons in parallel experiments expressing Kir2.1. While TeTx blocks synaptic vesicle release, Kir2.1 causes hyperpolarization of neurons via potassium channels.

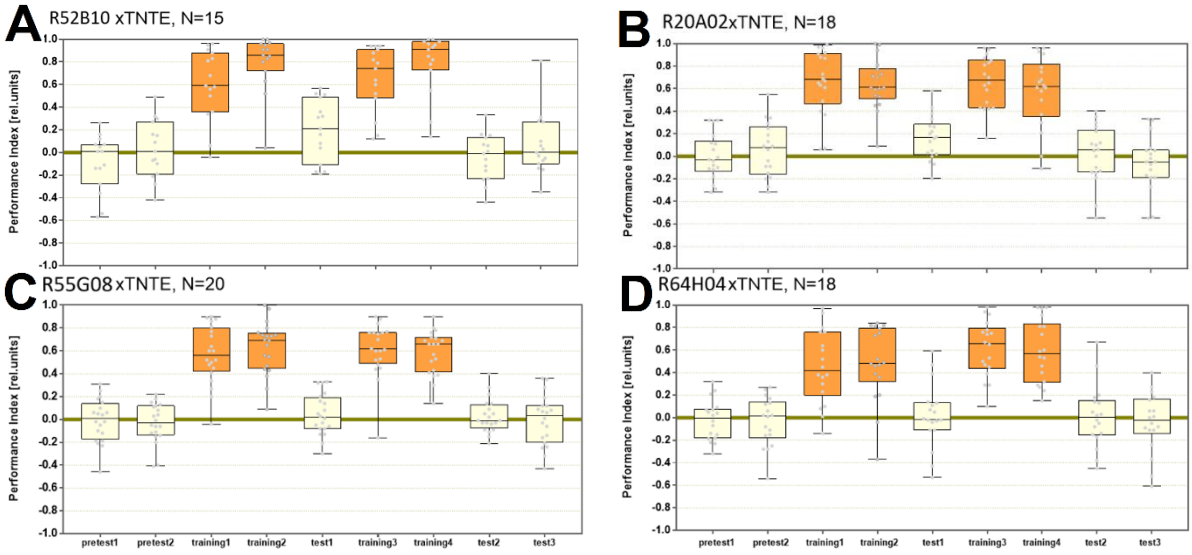

Figure 12: Reported learning defect of blocking local brain areas by Liu work group, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods, A, B: blocking of PCB. C,D: blocking of EB.

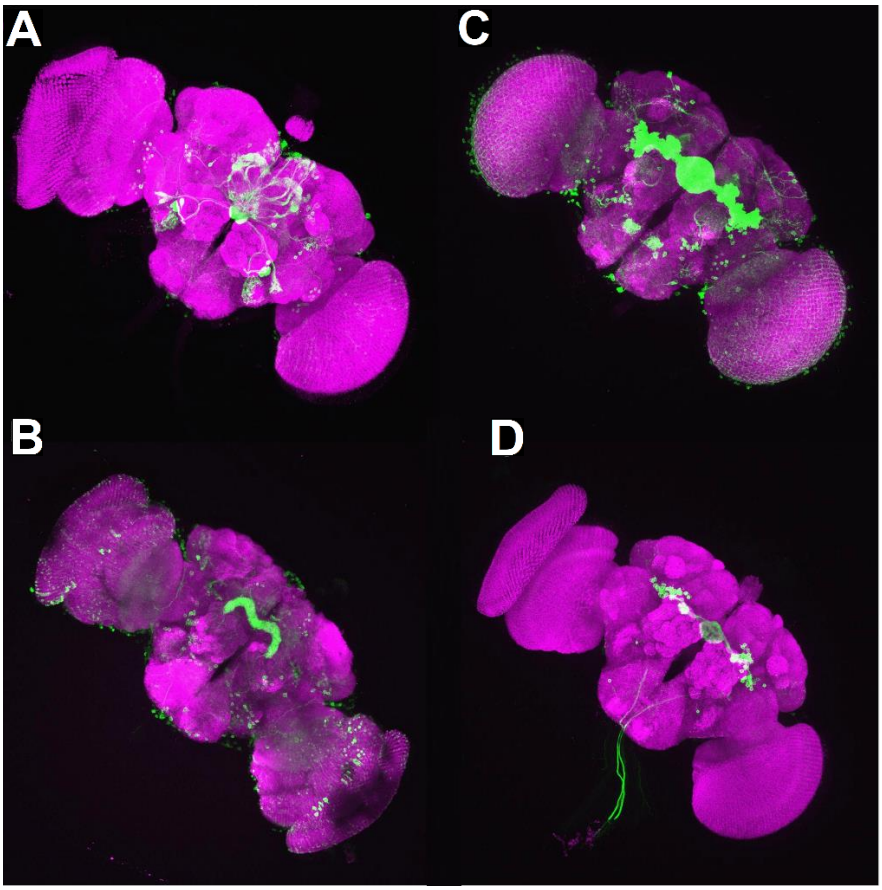

Figure 13: Expression pattern of the Gal4-lines: A: expression pattern of GMR52B10 (green), B: expression pattern of GMR55G08 (green), C: expression pattern of GMR20A02 (green), D: expression pattern of GMR64H04 (green). Image from flylight.com.

3.3.1 Blocking with TeTxG and Kir2.1

Due to unclear personal communication with Liu a different TeTx line, TeTxG, was used for the three following experiments.

3.3.1.1 Blocking of the ellipsoid body

GMR64H04 driver line was crossed to *UAS-TeTxG* or *UAS-Kir* to block the target neurons within the EB (Fig. 13D). Canton S (CS-TZ) flies were crossed to the driver line as control. Both experimental crosses showed increased PIs in the first test period after the last training and were significantly different from 0 (p = 1.91e-06 and p = 0.000322 respectively) (Fig.14, experimental sequence Fig. S10). The control cross showed increased PI in period 12 but was not significantly different from 0 (p = 0.0107). Blocking the expression pattern of *GMR64H04* with TeTxG or Kir2.1 did not lead to learning impairment.

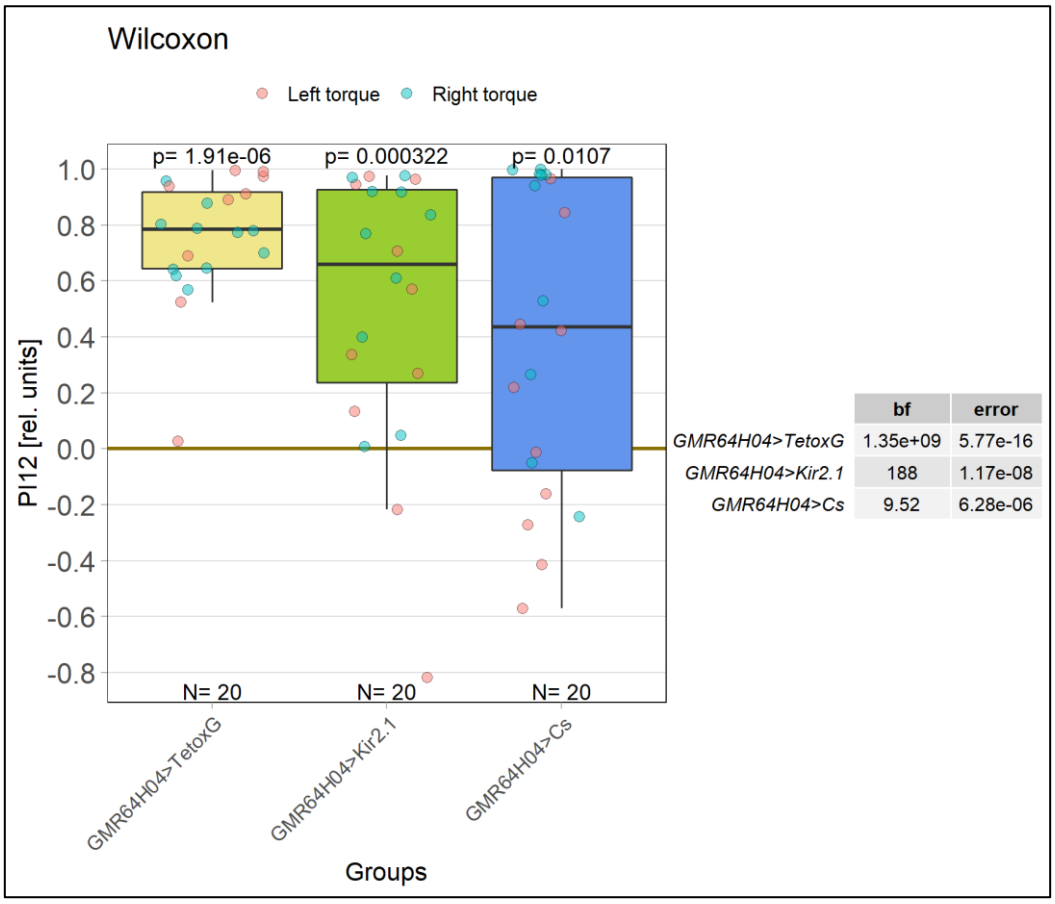

Figure 14: Blocking of the EB with TeTxG or Kir2.1. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: GMR64H04-Gal4>UAS-TeTxG, GMR64H04-Gal4>UAS-Kir2.1 GMR64H04-Gal4>CS-TZ Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

3.3.1.2 Blocking of the protocerebral bridge

GMR55G08 driver line expresses mainly in the PCB (Fig. 13B). It was crossed to the same effector lines and CS respectively. Corresponding to the previous experiment, the control cross did not show a significant difference from 0 in period 12 (p = 0.0441) (Fig. 15, experimental sequence Fig. S11). The PI was still increased, which would indicate the flies were still able to learn. Blocking the PCB with Kir2.1 did not lead to a learning defect. The PI was increased in the first period after training and was significantly different from 0 (p = 0.000447). As blocking the neurons within the PCB with TeTxG resulted in impaired flying performance of the flies, the experiment was stopped at a sample size of 16 flies. Thus, the results were not evidential. Although a reduced PI not different from 0 (p = 0.0355) during testing could be observed, no conclusion can be made. Flies showed poor vitality. About 2/3 of the flies had to be discarded before or during the optomotor adjustments, as they stopped flying constantly.

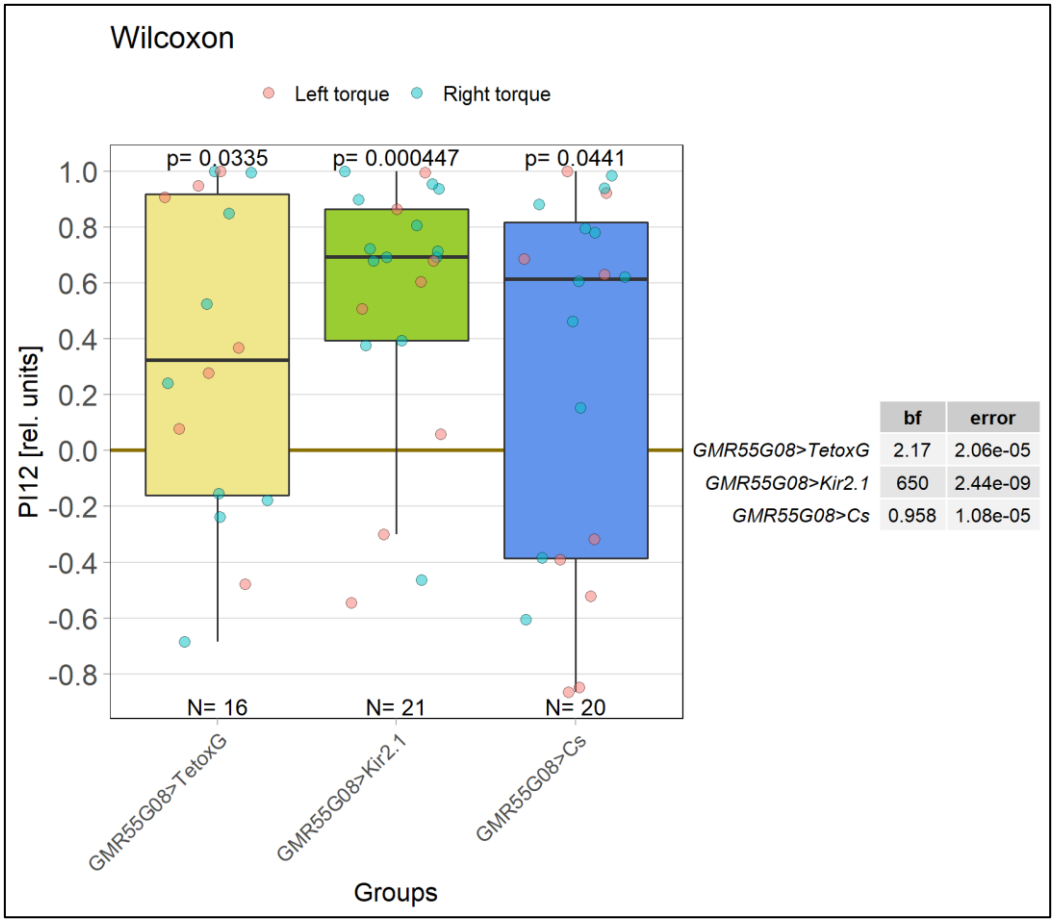

Figure 15: Blocking of the PCB with TeTxG or Kir2.1. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: GMR55G08-Gal4>UAS-TetxG, GMR55G08-Gal4>UAS-Kir2.1 GMR55G08-Gal4>CS-TZ. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

GMR52B10 driver line express mainly in the PCB (Fig. 13A). Crosses were performed corresponding to the previously described experiments. Likewise, the control cross was not significantly different from 0 in period 12 (p = 0.0365) (Fig. 16, experimental sequence Fig. S12). Both experimental crosses showed increased PIs in the first period after training and were significantly different from 0 (p = 0.00182 and p = 6.68e-05 respectively). Blocking the PCB with TeTxG or Kir2.1 in the expression pattern of *GMR52B10* did not lead to a learning defect.

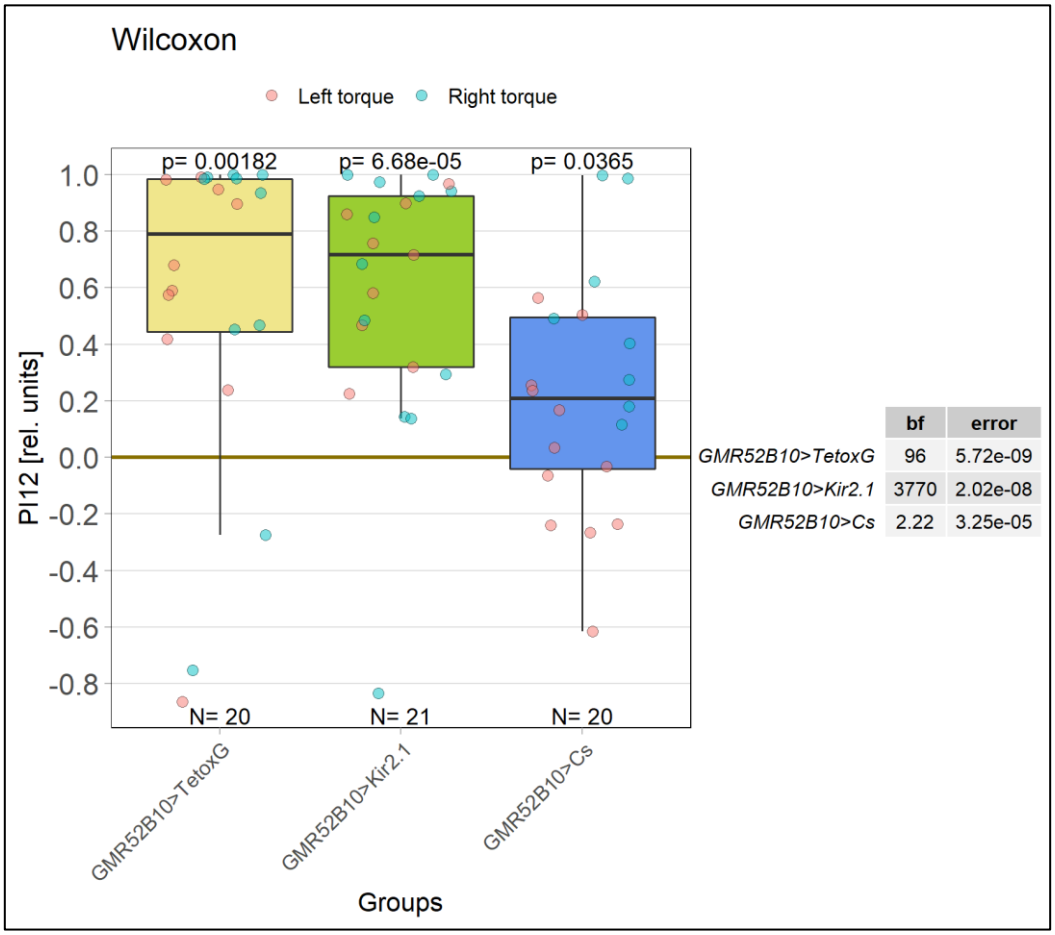

Figure 16: Blocking of the PCB with TeTxG or Kir2.1. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: GMR52B10-Gal4>UAS-TetxG, GMR52B10-Gal4>UAS-Kir2.1 GMR52B10-Gal4>CS-TZ. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

3.3.2 Blocking with TeTxE and Kir2.1

Having the discrepancies between the obtained results and those of the collaborative group, subsequent discussion revealed a difference in the *UAS-TeTx* lines used for blocking candidate brain areas in both labs. Therefore, the experiments were repeated using *UAS-TeTxE* with a weaker expression level including one further driver line.

3.3.2.1 Blocking of the ellipsoid body

GMR20A02-Gal4 expresses in the EB (Fig. 13C). It was crossed to CS as control. Crossings to *TeTxE* and *Kir2.1* effector lines were used as experimental groups with blocked neuronal activity within the brain area. Here, the control flies showed normal learning behaviour (Fig. 17, experimental sequence Fig. S13). The PI was increased in the first period after training and was significantly different from 0 ($p = 0.000483$). Blocking the EB with Kir2.1 did also not lead to an impairment. The PI in period 12 was increased and was significantly different from 0 ($p = 0.00151$) as well. Blocking the area with TeTxE led to learning impairment. The PI of the first test period after training was not significantly different from 0 ($p = 0.0883$).

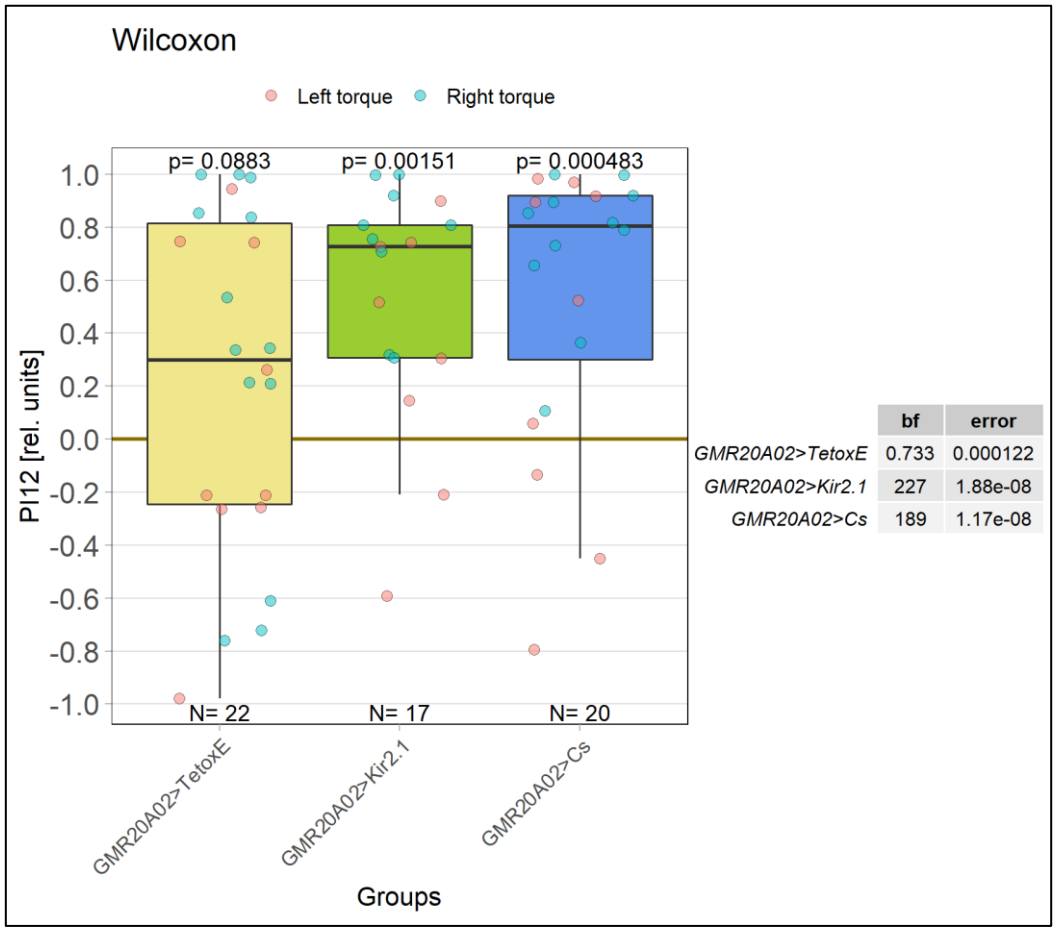

Figure 17: Blocking of the PCB with TeTxE or Kir2.1. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: GMR20A02-Gal4>UAS-TetxE, GMR20A02-Gal4>UAS-Kir2.1 GMR20A20-Gal4>CS-TZ. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

3.3.2.2 Retest of previous lines

Since blocking of the EB using TeTxE had an effect on self-learning the three previously tested lines were retested with TeTxE. A cross with CS was used as control (Fig. 18, experimental sequence Fig. S14). All the three experimental crosses showed increased PIs in the test periods. Only for *GMR55G08* a significant difference from 0 could be observed in the first test period after training (p = 0.00384). The other two experimental crosses were not significantly different from 0, indicating a learning defect (p = 0.0172 and 0.068 respectively).

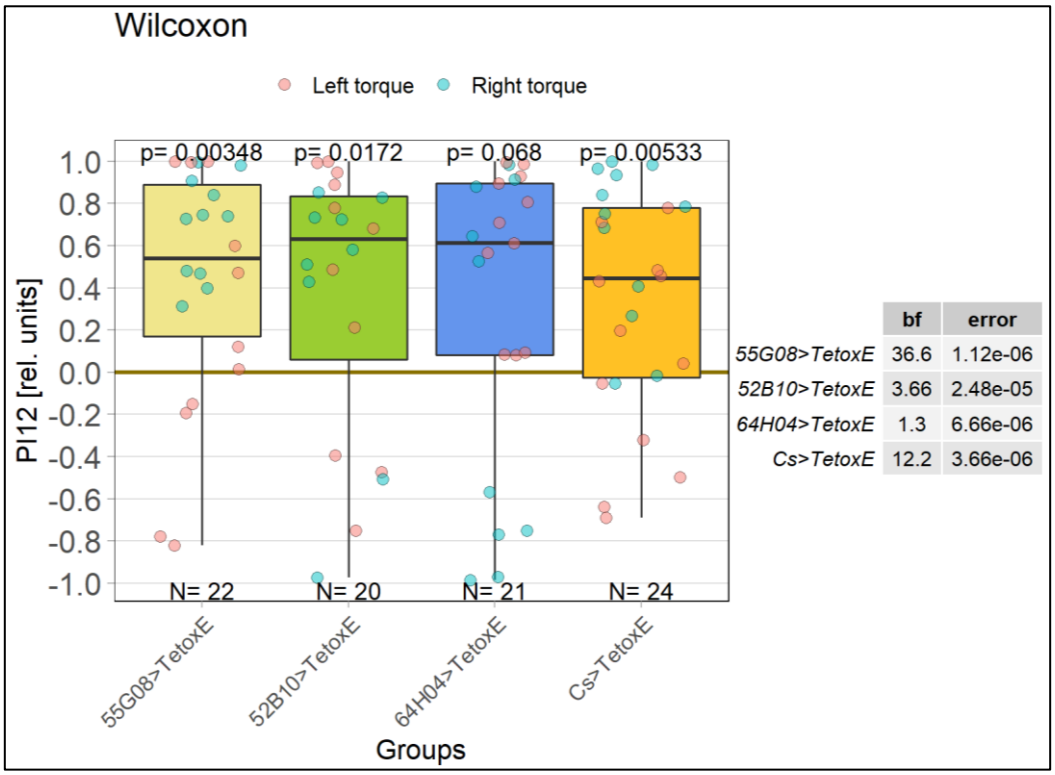

Figure 18: Retesting of the previous three lines, blocking with TeTxE . Performance index (PI) for the first test period after the last training. Y-axis: PI of periods 12, x-axis: tested groups: GMR55G08-Gal4>UAS-TetxE, GMR52B10-Gal4>UAS-TetxE, GMR64H04-Gal4>UAS-TetxE, CS-TZ>UAS-TetxE >. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

Comparing the blocking with TeTxE to Kir2.1 reveals a clear difference. While all the lines blocked with Kir2.1 seem to be able to learn, only *GMR55G08* shows learning behaviour when blocked with TeTxE.

3.4 *PKC* manipulation

Brembs and colleagues showed an involvement of the *PKC* family in self-learning behaviour (Brembs & Pendel, 2008; Colomb & Brembs, 2016). A possible interaction with *FoxP* was therefore considered.

3.4.1 *PKCi* expression

Overexpression of PKC pseudosubstrate (*PKCi*) blocks all PKC isoforms. To reproduce previous results and further investigate the role of *FoxP*, three groups were tested: *PKCi* expression in all neurons during development, *PKCi* expression in adult flies by using the *Gal4* inhibitor Gal80, and *PKCi* expression limited to *FoxP-iB* positive neurons. All flies were raised at 18°C due to the temperature sensitive nature of Gal80. Prior to experiment, flies received a 35°C heat shock for 4 hours. Constant expression of *PKCi* during development did not lead to learning impairment (Fig. 19, experimental sequence Fig. S15). The PI during testing was increased compared to the pretest and was significantly different from 0 ($p = 7.41e-05$). When limiting the expression to adult stage a learning impairment could be observed. The PI during the test period was not different from 0 ($p = 0.123$). Similar effect could be observed, when limiting *PKCi* expression to *FoxP-iB* positive neurons. This cross was also not able to perform in the self-learning task and did not show memory expression in a test situation ($p = 0.0583$). Noteworthy, the expression in *FoxP-iB* was not limited to adult flies. Even though *PKCi* was already expressed during development, the animals were unable to compensate, unlike when it is expressed in all neurons. As *PKCi* expression in *FoxP-iB* positive neurons impaired operant self-learning a link between *FoxP* and *PKC* could be suggested.

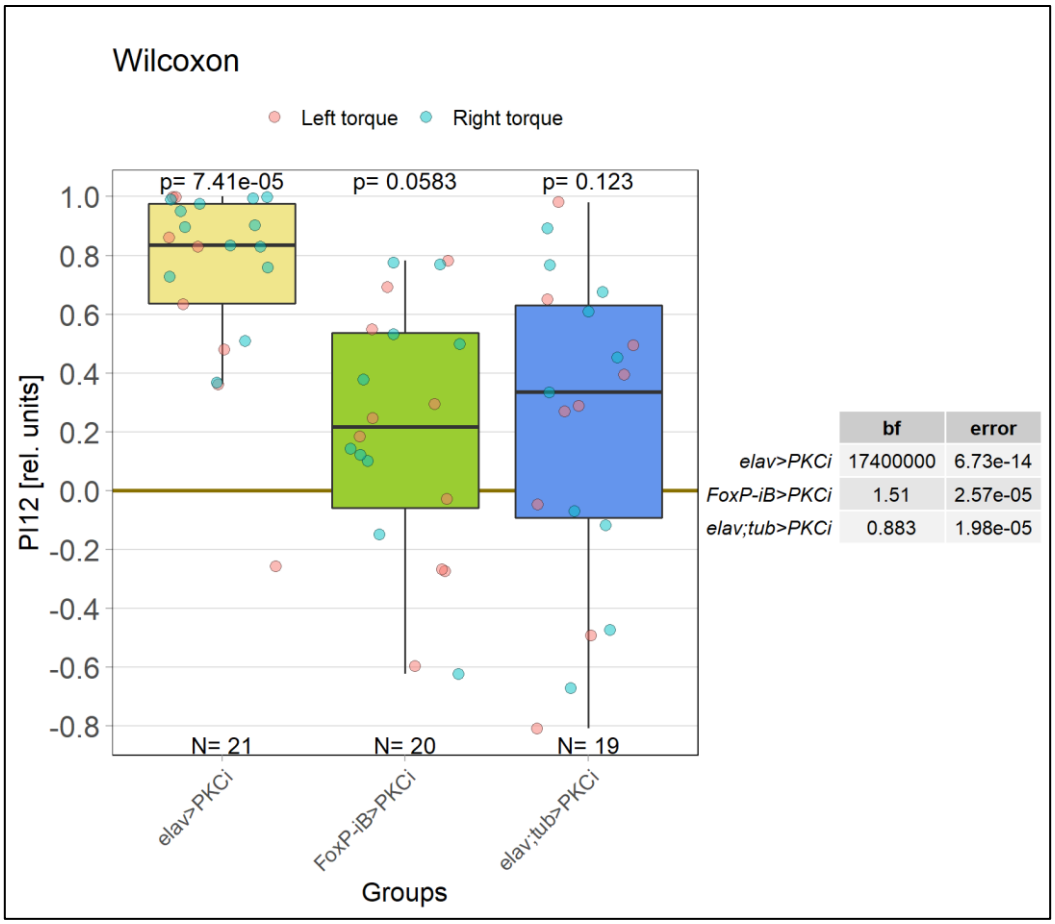

Figure 19: Expression of PKCi in all neurons during development or in adult flies, or in FoxP-iB neurons. Performance index (PI) for the first period after the last training. Y-axis: PI of period 12, x-axis: tested groups: elav-Gal4>UAS-PKCi, FoxP-iB-Gal4>UAS-PKCi, elav-Gal4;tubGal80>UAS-PKCi. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

3.4.2 Knockout of *aPKC* and *PKC53e*

Since five different *PKC* isoforms are expressed in *Drosophila*, it was aimed to dissect which of those affect learning behaviour. Using RNAi posed issues in past studies. Therefore, it was not possible to narrow down possible candidate genes previously (Colomb & Brembs, 2016). Due to the recent tool development and implementation of the CRISPR/Cas9 system it was possible to test knockout of two different PKCs, *aPKC* and *PKC53e*. To excluded developmental effects temporal knockout via the gene-switch system was implemented for this experiment. Flies were raised under normal conditions (see material and methods section). Freshly hatched flies were transferred on fly food containing the steroid hormone RU486 two days before gluing.

Knocking out *PKC53e* in all neurons of adult flies did not result in learning impairment (Fig. 20, experimental sequence Fig. S16). The PI after training was increased and significantly different from 0 (p = 0.000583). A knockout of *aPKC* in all neurons lead to a decreased learning ability. Although the PI of period 12 still seemed high it was not significantly different from 0 (p = 0.0105). Thus, *aPKC* is a potential candidate for modulating operant self-learning whereas *PKC53e* seems not to be involved

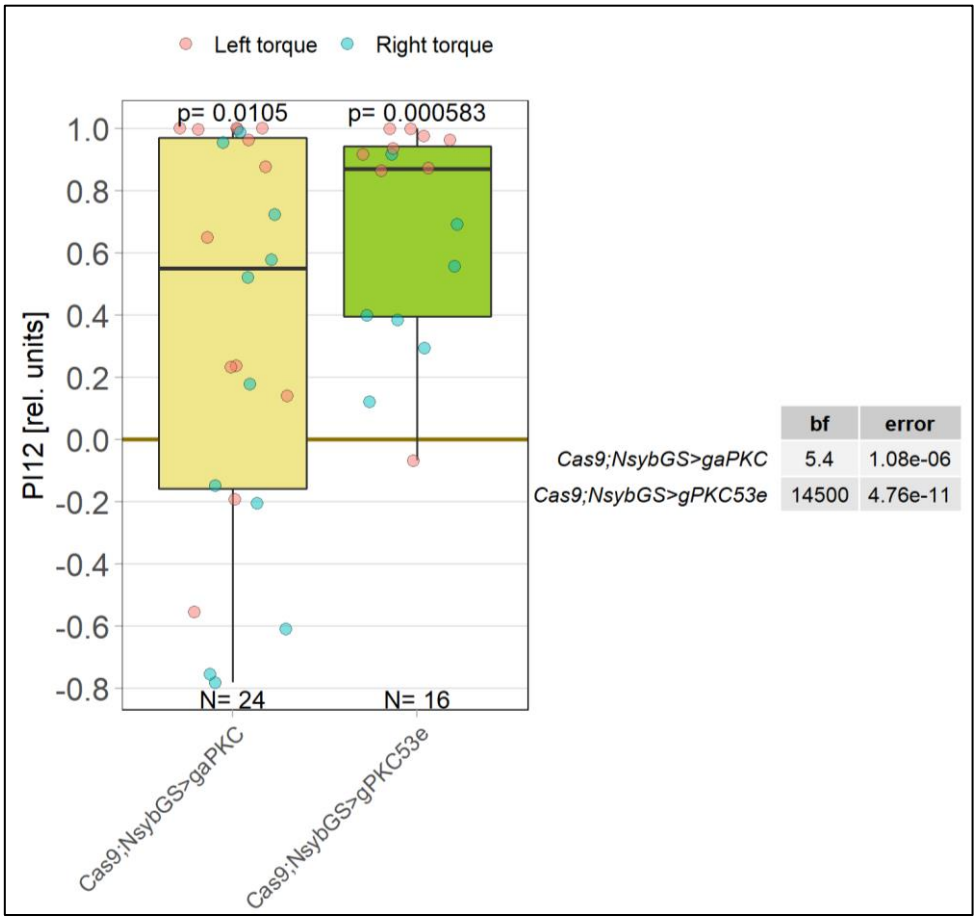

Figure 20: Knockout of aPKC or PKC53e in all neurons in the adult fly. Performance index (PI) for the first training period after the last training. Y-axis: PI of period 12, x-axis: tested groups: UAS-Cas9;NsybGS-Gal4>gaPKC, UAS-Cas9;NsybGS-Gal4>gPKC53e. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

3.4.3 Local *aPKC* knockout

In order to unravel a potential interaction between *FoxP* and *aPKC*, *aPKC* was specifically knocked out in all *FoxP-iB* positive neurons during development. Further, previous experiments have demonstrated an involvement of motor neurons in operant self-learning (Colomb and Brembs, 2016). Therefore, *aPKC* was knocked out in all motor neurons during development as well. Due to better flying performance of the flies, *380-Gal4* driver line was chosen rather than *D42-Gal4* driver line. *aPKC-UAS-Cas9* flies were crossed to Wtb as a control (Fig. 21, experimental sequence Fig. S17) such that *aPKC* expression was not altered. In the experiment they showed an increased PI after training and were significantly different from 0 ($p = 0.0016$). Animals missing *aPKC* in all motor neurons were impaired in their self-learning ability. The PI was not increased after training and was not different from 0 ($p = 0.648$). The same effect can be observed if the knockout of *aPKC* is limited just to the *FoxP-iB* positive neurons. Their PI was not increased after training and was not different from 0 ($p = 0.104$) as well.

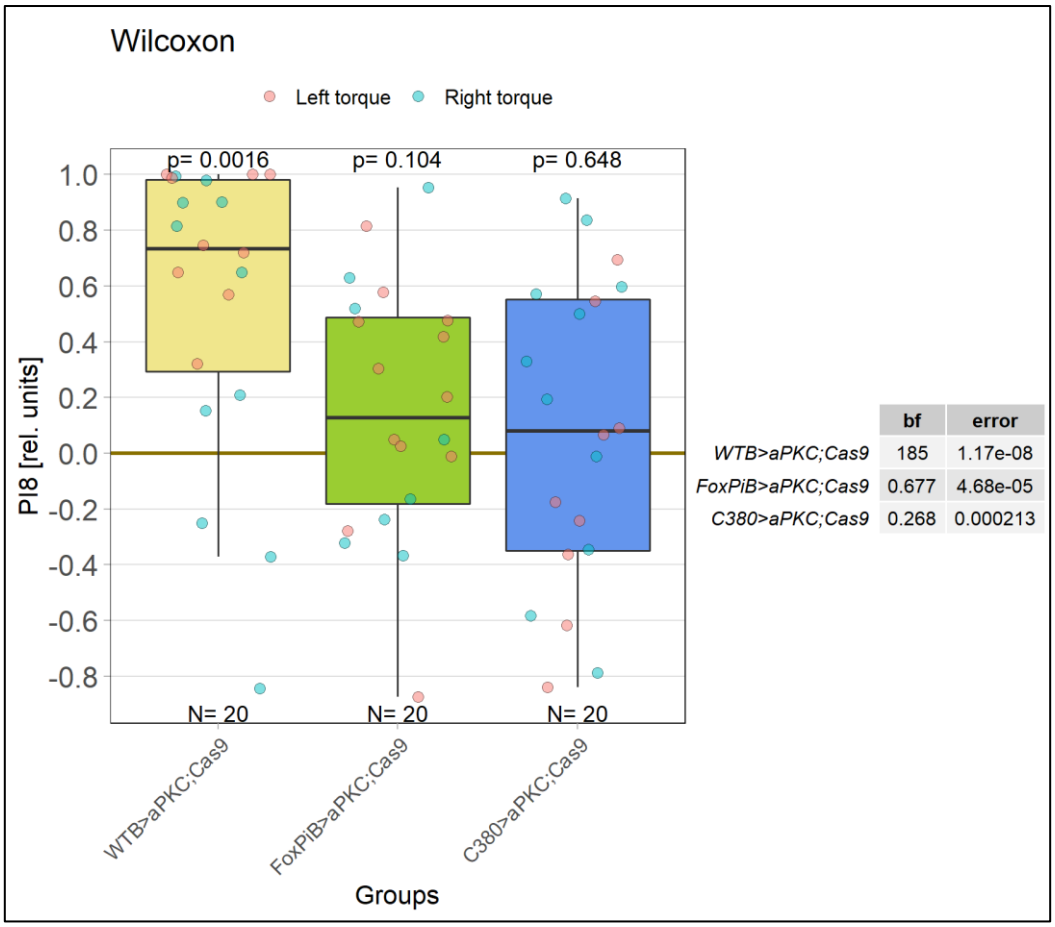

Figure 21: Knockout of aPKC in all motor neurons or FoxP-iB positive neurons. Performance index (PI) for the first training period after the last training. Y-axis: PI of period 8, x-axis: tested groups: WTB>gaPKC;UAS-Cas9, FoxP-iB-Gal4>gaPKC;UAS-Cas9, C380-Gal4>gaPKC;UAS-Cas9. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

3.4.4 *aPKCΔ* developmental expression

It was shown that the *aPKC* knockout causes a learning defect. The subsequent question was whether *aPKC* overexpression would have a positive effect on self-learning. So, the effector line *UAS-aPKCΔ* was used to upregulate *aPKC* expression. aPKCΔ is a truncated from without the normal regulatory domain. It is not affected by the normal regulatory mechanism of aPKC. It is therefore continuously active, leading to a higher aPKC activity. For this experiment the same driver lines were used as in the previous experiment. Further, the motor-neuron line *D42-Gal4* was added (Fig. 22, experimental sequence Fig. S18).

Expressing *aPKCΔ* already during development had severe effect on the flying performance of the animals. If *aPKCΔ* was expressed in motor neurons flies showed flying deficits, so the desired sample size could not be obtained. Due to low number of flies and poor flight performance no real conclusion could be made regarding the learning ability. Animals expressing *aPKCΔ* in *FoxP-iB* positive neurons seemed to show learning behaviour. The PI was increased after training but was not significantly different from 0 (p = 0.0436). The control cross did not show a significant difference from 0, even though the PI was increased (p = 0.0703). Expressing *aPKCΔ* in the motor neurons during development had severe effect on the vitality of the flies. *aPKCΔ* expression in *FoxP-iB* neurons had milder effect, so flies were still able to perform the experiment.

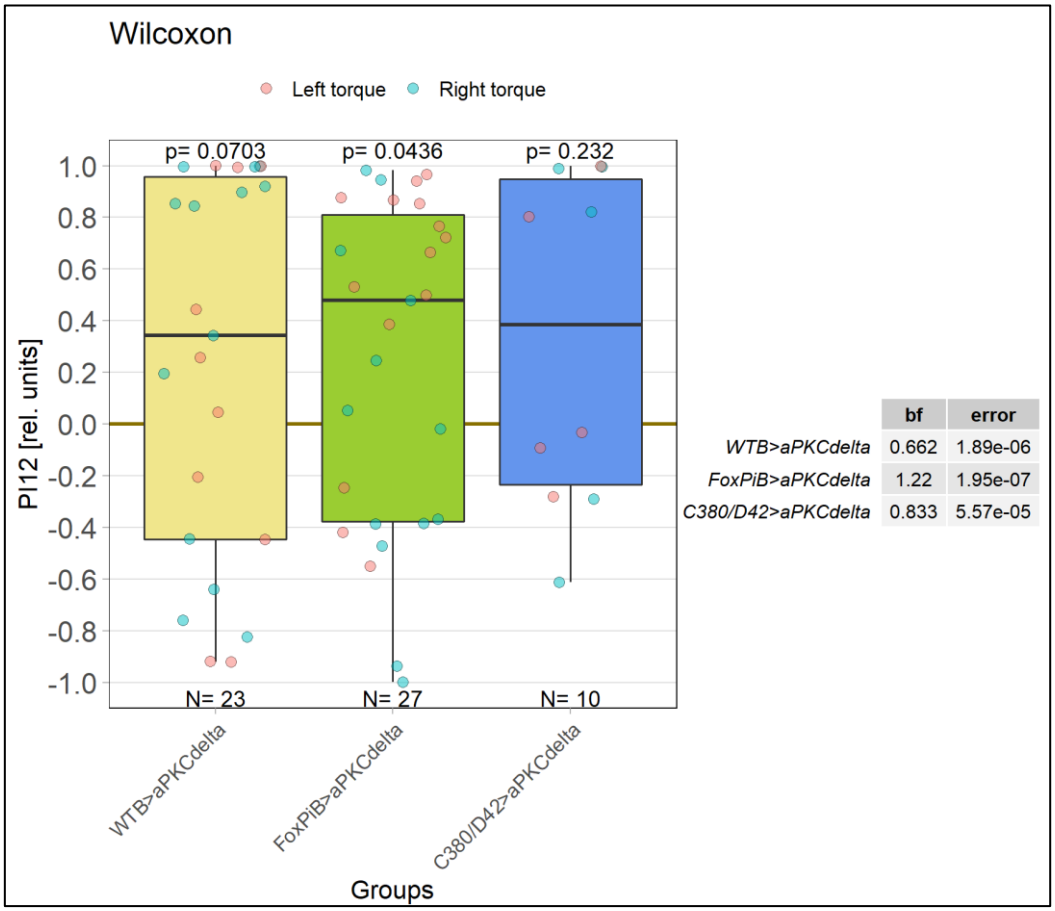

Figure 22: Expression of aPKCΔ in FoxP-iB positive or motor neurons. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: WTB>UAS-aPKCΔ, FoxP-iB-Gal4>UAS-aPKCΔ, C380-Gal4/D42-Gal4>UAS-aPKCΔ. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

To test whether reduction of the training periods would affect learning, the same crosses were used. The time of each experimental period was shortened from two to one minute. Thereby, flies would only get four minutes of training in total. Flies that are undertrained this way should not be able to learn. Since the lack of *aPKC* led to learning impairment overexpression might lead to an improvement.

With only half the experimental duration it was also possible to test enough of the flies with *aPKCΔ* expression in the motor neurons. An effect in the learning performance could be observed during the intermediate test (period 9) (Fig. 23B). In contrast to the control cross the PIs of the two experimental groups were significantly different from 0 ($p = 0.00472$, $p = 0.00144$ respectively). All three groups showed slightly increased PIs in the first test period after training (Fig. 23A, experimental sequence Fig. S19). But no group showed a significant difference from 0.

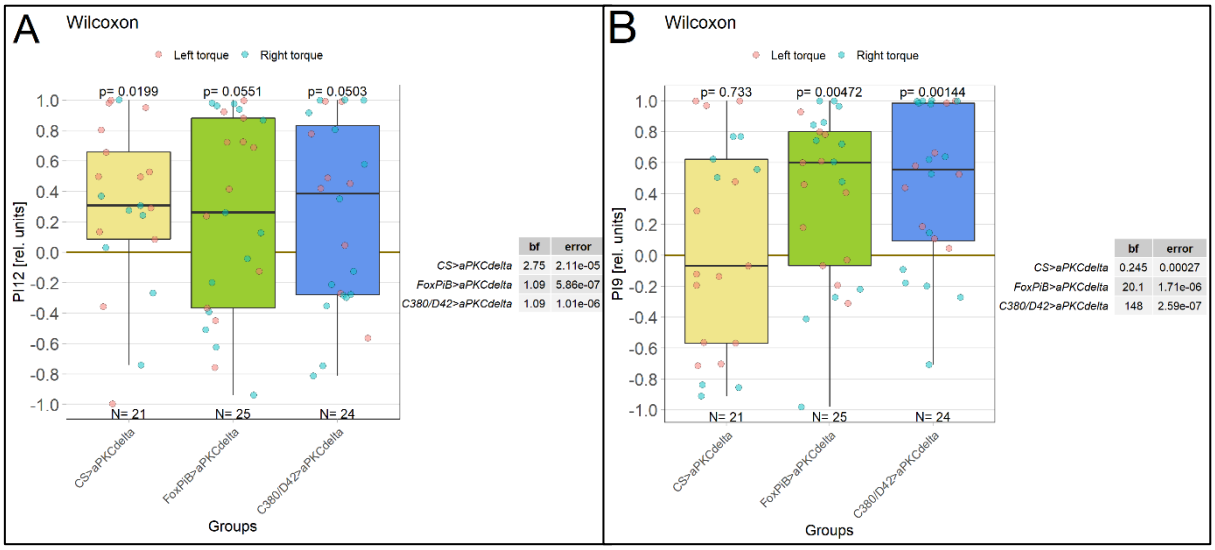

Figure 23: Expression of aPKCΔ in FoxP-iB positive or motor neurons, half the period duration (1 min). A: Performance index (PI) for the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: CsTs>UAS-aPKCΔ, FoxP-iB-Gal4>UAS-aPKCΔ, C380-Gal4/D42-Gal4>UAS-aPKCΔ. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics. B: PI of the test period between training periods, Y-axis: PI of period 9, x-axis: tested groups: CsTs>UAS-aPKCΔ, FoxP-iB-Gal4>UAS-aPKCΔ, C380-Gal4/D42-Gal4>UAS-aPKCΔ. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

3.4.5 *aPKCΔ* adult expression

To exclude developmental effects of *aPKC* overexpression, the gene-switch system was used. ***aPKCΔ*** was expressed panneuronally. Experimental flies were fed with a steroid hormone to activate the expression of the transgene. The control group was genetically identical but was not placed on a steroid hormone (Fig. 24). Although the PI of the control group was increased in the first test period after training, it was not significantly different from 0 (p = 0.0149). The learning performance of the experimental flies after training was increased, but not significantly different from 0 (p = 0.0362). The PI during the last test (PI13) is also increased, indicating that flies were still able to learn (Fig. S20).

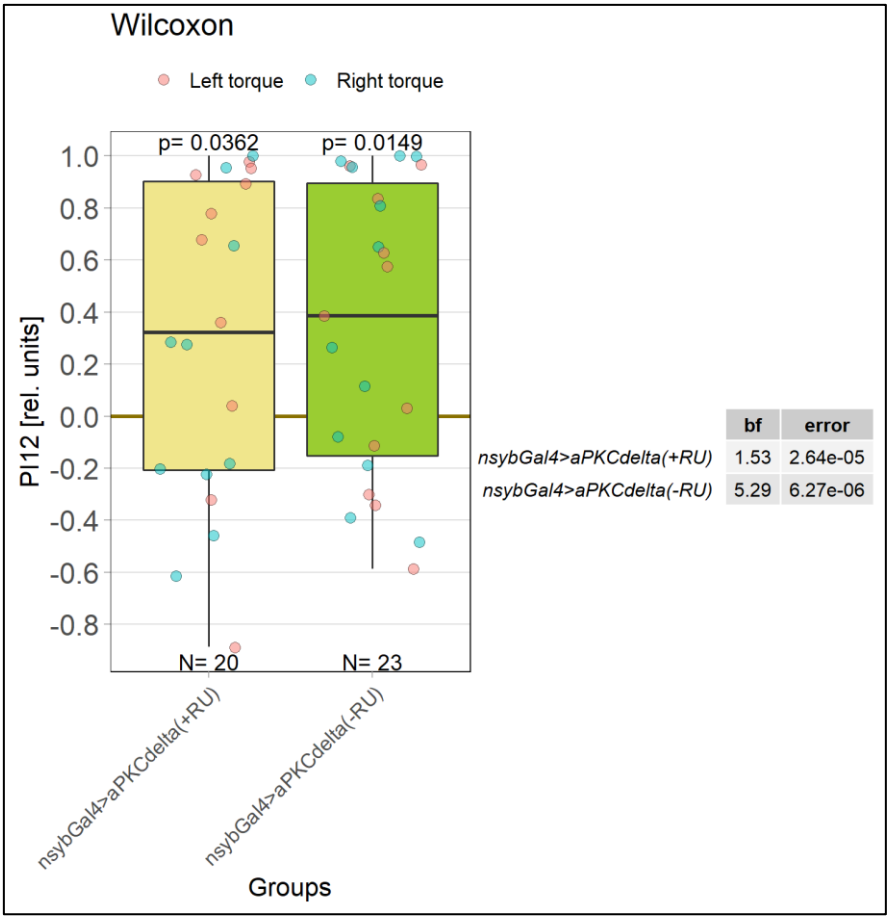

Figure 24: Expression of aPKCΔ in FoxP-iB positive or motor neurons. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: nsybGs-Gal4>UAS-aPKCΔ with and without RU. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

Using the same two groups, the experimental time was shortened such that flies were trained for 4 minutes in total (Fig. 25, experimental sequence Fig. S21). Flies with no *aPKCΔ* expression showed no significant difference from 0 after training (p = 0.0291). *aPKCΔ* expression in all neurons resulted in increased PI in the first test period after training (p = 0.00034). This would indicate that an overexpression of *aPKC* is improving the learning ability of the flies. Thus, flies seemed to be able to still perform in the self-learning task, while being undertrained.

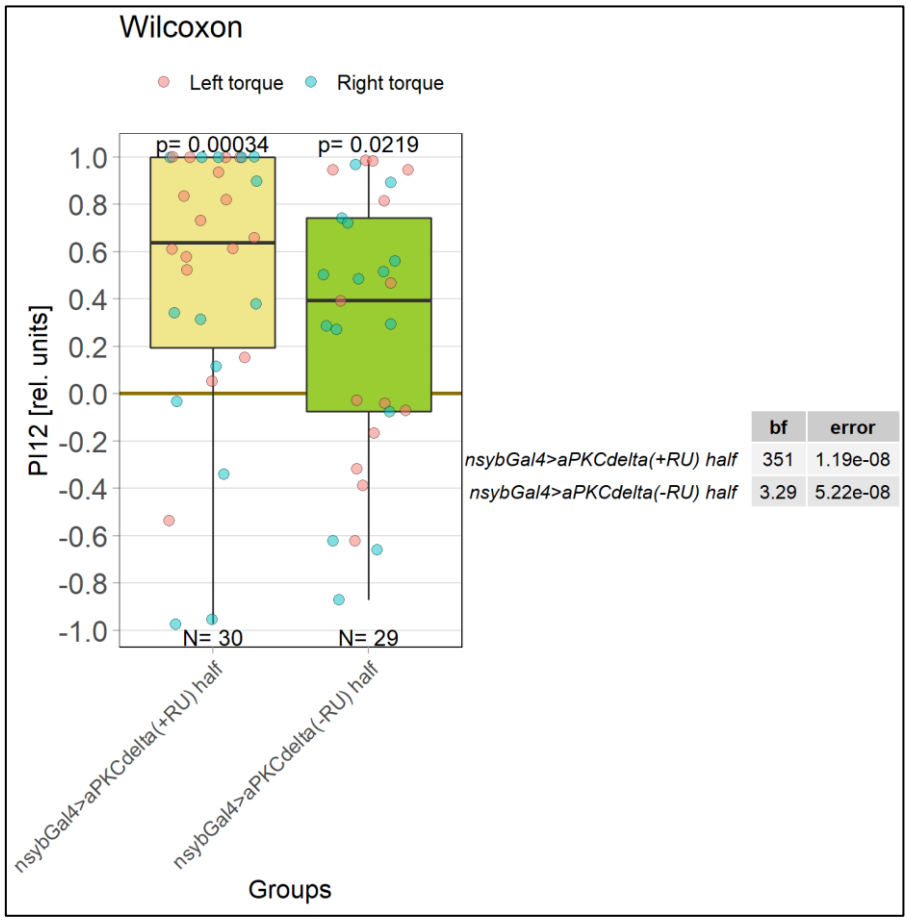

Figure 25: Expression of aPKCΔ in all neurons in adult flies, half the period length (1 min). Performance index (PI) of the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: nsybGs-Gal4>UAS-aPKCΔ with and without RU. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

3.4.6 Overlap of *FoxP* and *aPKC*

Based on the behavioural experiments, a link between *FoxP* and *aPKC* was suggested. *FoxP* and *aPKC* positive neurons were labelled with fluorescent proteins and brains and ventral nerve cords (VCN) were dissected to identify potential expression overlap. A colocalization in the expression pattern within the brains was not observed (Fig. 27A). In the VNC colocalization could be detected (Fig. 27A,B). A comparison with Maniates-Selvin et. al. 2020 revealed that wing neurons exhibit *FoxP* and *aPKC* gene expression (Fig. 26A-D). A prominent expression of *FoxP* was detected in the last segment of the VCN, the abdominal neuromere (ANm). Here, all abdominal neuromers are fused together.

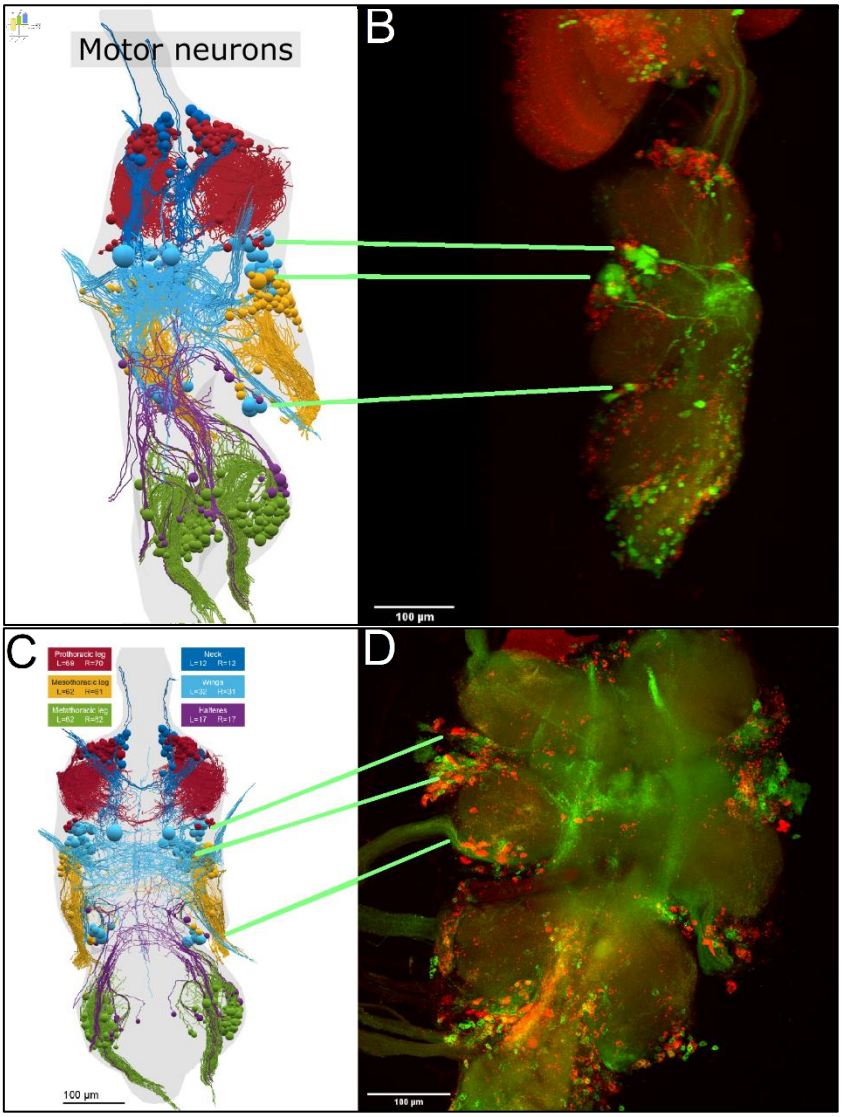

Figure 26: Anatomy of FoxP and aPKC in the adult VNC. Panel A,C: reconstruction of motor neurons of the VCN from Maniates-Selvin et. al. 2020. Panel B: Adult VCN, green D42-Gal4>CD8::GFP, red FoxP-LexA>CD8::RFP. Green lines point towards corresponding areas. Panel D: Adult VCN, green aPKC-Gal4>CD8::GFP, red FoxP-LexA>CD8::RFP. Green lines point towards corresponding areas.

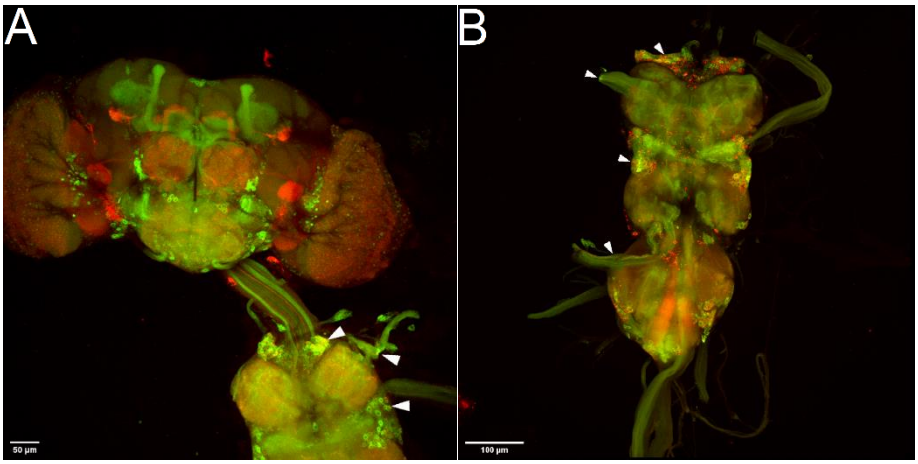

Figure 27: No coexpression of FoxP and aPKC in the adult brain, coexpression in the VNC. Panel A: Adult brain with part of VCN, green aPKC-Gal4>CD8::GFP, red FoxP-LexA>CD8::RFP, white arrows indicate examples for colocalization. Panel B left:: Adult VCN, green aPKC-Gal4>CD8::GFP, red FoxP-LexA>CD8::RFP, white arrows indicate examples colocalization.

3.4.7 *PKC* summary

Out of the five *PKCs* isoforms expressed in *Drosophila*, *aPKC* was shown to be involved in operant self-learning. Manipulation of *PKC53e* had no effect (Fig. 21). Knocking out *aPKC* in all neurons in the adult flies or during development in motor- or *FoxP-iB* positive neurons led to learning impairment (Fig. 19 and Fig. 21). Overexpression of *aPKC* with ***aPKCΔ*** seemed to improve the learning ability of the flies (Fig. 25).

3.4.8 *BAZ* knockout

aPKC was reported to form the PAR complex together with Par-6 and Bazooka (Baz). This complex is involved in several pathways, with Hedgehog Signaling Pathway (HH) and Hippo signaling pathway (HPO) being two prominent ones. Since *aPKC* seems to be necessary for the self-learning ability of flies, the involvement of this complex for operant self-learning was investigated. A different part of the complex, *baz,* was therefore targeted (Fig. 28, experimental sequence Fig. S24). Using the gene-switch system once more, *baz* was knocked out in all neurons of adult flies. Freshly hatched *Drosophila* were kept for 48 hours on food containing RU486, the control group was kept on food without supplement.

Both groups showed an increased PI in the first test after training and were significantly different from 0 (p = 6.56e-06 and p = 0.000808 respectively). The experimental group seemed to perform even better than the control. Thus, *baz* is most probably not necessary for operant self-learning in adult flies.

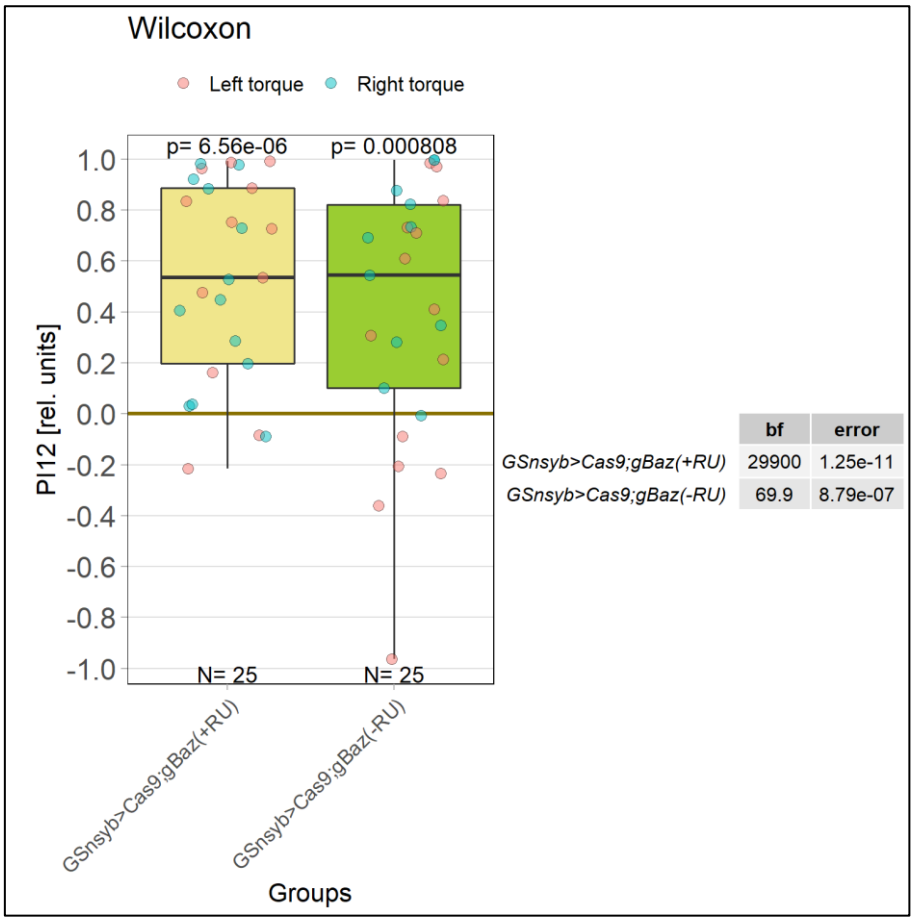

Figure 28: Knockout of baz in all neurons of the adult fly. Performance index (PI) for the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: nsybGs-Gal4>UAS-Cas9;gBaz with RU and without. Each point representing one fly. Wilcoxon test against 0 with bayesian statistics.

4. Discussion

FoxP was show to be important for vocalisation in a variety of species (Fisher and Scharff, 2009). Conceptually, speech learning and operant self-learning have the same mechanism, providing no external cue to the animal. Therefore, an operant self-learning paradigm was also used in this study. Many studies dissected the effect of *FoxP* in *Drosophila* (Castells-Nobau et al., 2019; Co et al., 2020; Lai et al., 2001; Palazzo et al., 2020; Villalobos et al., 2021; Zhang et al., 2002). Most of them investigated temporal and/or spatial parameters. Only few studies looked at learning behaviour. Mendoza et al., 2014 showed that *FoxP* mutants were specifically impaired in operant self-learning, while still being able to perform a world learning task.

4.1 Temporal *FoxP* manipulations

It was not possible to test *FoxP* knockout during development, so it was limited to the adult flies. Since *FoxP* mutants are reportedly impaired in operant self-learning, it was assumed those transgenic *FoxP* manipulations would also lead to learning defects. It was shown that even after loss of *FoxP* expression in adult flies, animals were still able to learn. This is surprising, but points towards the developmental role of *FoxP*. Further, Palazzo et al. 2019 were only able to show severe motor impairments by knocking out *FoxP* during development. When *FoxP* was knocked out only in adult flies no differences could be observed. In zebra finches, it was shown that continuous *FoxP* expression is necessary in adults to maintain the singing ability (Day et al., 2019). We therefore aged the flies after the knockout and tested seven and 14-day old flies. Since *FoxP* is a transcription factor, a temporally shifted effect of the manipulation was considered. Flies were still able to learn after seven days without *FoxP* but were impaired after 14 days. However, seven days should have been sufficient to exclude any lingering effects. This could point to long half-life of the gene product. On the other hand, *FoxP* expression could be crucial for the maintenance of the learning ability, like reported in birds (Day et al., 2019). Gene expression is reduces with age of flies (Davie et al., 2018). So, age-dependent decrease of learning performance could be based on reduced *FoxP* expression.

The strong motoric impairments of flies with a developmental *FoxP* knock-out points towards the importance of this gene for development. In addition, the result suggests the need of maintained FoxP expression in the adult flies for operant self-learning.

4.2 Local *FoxP* manipulations

FoxP is expressed in many different brain areas in *Drosophila* (Palazzo et al., 2020). So far, it was not known where *FoxP* expression is needed for self-learning. Therefore, *FoxP* was knocked out in different parts of the fly brain, using the CRISPR/Cas9 system. None of the tested manipulations led to impairment in the self-learning ability of flies. Conditional local knockout of *FoxP* was shown to lead to motor defects in mice (French et al., 2019). It was assumed that the knockout was successful. As a control male *Cas9gFoxP* parents of each cross were paired with *elav-Gal4* females. Since the phenotype of a developmental *FoxP* knockout is quite severe and distinct, the validity of the tested flies could easily be checked (Palazzo et al., 2020). Due to the lack of FoxP-antibody this control was used instead. This quality control was used for every cross that involved the *Cas9gFoxP* line. It should be therefore possible to exclude an error with the *FoxP* knockout. Two possible explanations come to mind. *FoxP* could not be important in the targeted brain regions for operant self-learning. It is not known in which brain areas *FoxP* gene expression is necessary for this learning behaviour. The gene is expressed in many parts of the fly brain and could serve a different function. A second explanation could be the ability of flies to compensate for the loss of *FoxP*. Since the knock-out was local, *FoxP* was still expressed in other parts of the brain. This might be sufficient for the maintenance of the learning ability. High plasticity was reported in the fly brain (Heisenberg et al., 1995). Plasticity is the modification of neuronal circuits' functions by neuronal activity. Synaptic plasticity in particular is the activity-dependent change in strength or efficacy of transmission at the synapse (Citri and Malenka, 2008). Short term plasticity, lasting from milliseconds to minutes, is often the result of an accumulation of calcium at the postsynaptic nerve (Zucker and Regehr, 2002). In *Drosophila* plasticity was shown in the olfactory projection neurons to the mushroom body (Elkahlah et al., 2020). Moreover, in vertebrates, in addition to the brain, the spinal cord was discovered as an area with high plasticity (Wolpaw, 2010).

Using operant conditioning plasticity was even shown in simple spinal cord reflexes like the spinal stretch reflex or the H-reflex. Reward for higher or lower response, leads to corresponding increase or decrees of the reflex (Thompson et al., 2009; Wolpaw, 1987). The VNC in *Drosophila* serves a similar function as the spinal cord. Therefore, it would be reasonable to also observe plasticity in the VNC. When flies were developing without intact *FoxP* expression in different areas of the brain they were still able to perform the self-learning task. Since neuronal plasticity is important for learning, *FoxP* might not be important for plasticity in the fly brain. But gene expression can be found in the VNC. So, *FoxP* could mediate neuronal plasticity for operant self-learning in the VNC.

FoxP knockout in the PCB was shown to have some motoric impairment effects when flies were tested in Buridan's paradigm (Palazzo et al., 2020). Therefore, it is interesting that flies are still able to learn. *Drosophila* can show different behaviour dependent on the context (Ache et al., 2019; Card and Dickinson, 2008a, 2008b; Gorostiza et al., 2015). Depending on the state of the fly (e.g., sitting or flying) the same stimulus is causing a different response. In Buridan's paradigm flies are walking, in the DFS flight is required.

It was not possible to find a brain region were *FoxP* is needed for operant-self learning. Not all areas expressing *FoxP* could be tested. Saddle and vest were not targeted. No *Gal4* line with matching colocalisation could be obtained. However, these areas should not be disregarded for operant self-learning. It was also not possible to test the *FoxP* knockout in motor neurons in the learning task. Flies showed strong motoric impairments as reported in previous studies (Castells-Nobau et al., 2019; Palazzo et al., 2020). It was therefore not possible to test them in an experiment requiring constant flight. The motor neurons have been reported to be important for operant self-learning (Colomb and Brembs, 2016). The importance of *FoxP* for fine motor control, required in behaviours like vocalisation, was found in several studies across multiple species (Castells-Nobau et al., 2019; Fisher and Scharff, 2009; Fujita et al., 2008; Groszer et al., 2008; Kurt et al., 2012; Lawton et al., 2014). This underlies the conserved role of *FoxP*. It is reasonable to assume that flies without *FoxP* in motor neurons would be also impaired in their self-learning ability. This was underpinned by the results of the *PKC* manipulations.

4.3 PKC manipulations

The PKC protein family was reported to be important in different species for learning (Cai et al., 2011; Chatterji et al., 2020; Sakaguchi and Yamaguchi, 1997; Yoshida et al., 2003). It was not known, which isoforms are involved in operant self-learning. Colomb and Brembs, 2016, showed that *PKC* is an essential part of the self-learning mechanism in *Drosophila*. We were able to replicate the original results, the expression of *PKCi* in all neurons in adult flies blocked self-learning (Colomb and Brembs, 2016). However, flies were able to compensate for the expression of RNAi during their development and were still able to learn. In addition, limiting the expression to the *FoxP-iB* positive neurons also led to learning impairment. This indicates an interaction of *PKC* and *FoxP*. Expressing *PKCi* in all neurons or just in the subset of *FoxP-iB* positive neurons led to similar learning defect. Furthermore, *PKC* activity in *FoxP-iB* positive neurons was necessary for operant self-learning. Thus, a potential link between *PKC* and *FoxP* was further studied.

The use of RNAi lines was inconclusive in past experiments and it was not possible to narrow down the relevant *PKC* isoform (Colomb and Brembs, 2016). Using the CRIPR-Cas9 technique we were able show that *PKC53e* is not involved in operant self-learning. *aPKC* was noted as relevant *PKC* isoform for this learning behaviour. Indeed, previous studies suggested that *aPKC* is important for learning in *Aplysia* as well (Bougie et al., 2012; Hu et al., 2017). When *aPKC* was knocked out in all motor neurons flies were not able to learn anymore. The same effect could be observed when limiting the knockout to the *FoxP-iB* positive cells. It is sufficient to knock out this single isoform to impair the learning ability of flies. *aPKC* knockout in both experimental crosses seemed to have similar potent effect. Thus, potential role of *aPKC* in *Drosophila* for operant self-learning could be evinced. Secondly, it indicates a possible interaction of *FoxP* and *aPKC*. Expression of *aPKC* in *FoxP-iB* positive neurons was necessary for the flies to perform the learning task. Moreover, involvement of the motor neurons in this learning task could be demonstrated. This is in accordance with the results from Colomb and Brembs 2016. They could show learning defects by expressing *PKC-RNAi* in motor neurons. However targeting only the subset of *FoxP-iB* positive neurons lead to similar learning impairments.

Further, an improving effect of *aPKC* overexpression could be demonstrated. Upregulation of *aPKC* using ***aPKCΔ*** resulted in memory formation after half of the training time while wildtype flies showed no learning behaviour. Wildtype flies would not be able to learn with this form of undertraining. The PI was still increased and higher than expected. This was likely due to high laser intensity. The experiment could be reproduced by a student in blind (Fig S22, S23). Likely, due to the lower laser intensity the control flies showed no increased PI. Even though the experimental cross showed no significance, this was mainly due to a negative PI during the pretest. It therefore seems that *aPKC* improves the learning ability of flies, since the results could be reproduced by a second experimenter.

The experiment expressing ***aPKCΔ*** during development was inconclusive. None of the groups showed learning. The control group had no ***aPKCΔ*** expression and should behave like wildtype flies. The experimental line expressing ***aPKCΔ*** also showed no learning behaviour. The control cross did not learn, so no conclusion can be made. Since *aPKC* plays an important role for cell polarity it is conceivable that such developmental manipulations would have a wide variety of unintended effects on the flies.

PKMζ is a constantly active form of *aPKC* in vertebrates (Sacktor et al., 1993). Expressing the mouse aPKMζ in *Drosophila* leads to enhanced memory. In addition, chemical blocking of aPKMζ inhibited memory but not learning (Drier et al., 2002). Classical odour conditioning was used in this study. Like *FoxP, aPKC* is therefore not needed for world-learning. If a self-learning paradigm would have been used in the study, the authors would have likely observed a learning defect similar to the *aPKC* knockout. The expression of aPKMζ and aPKCΔ should have comparable effects. We would expect flies with ***aPKCΔ*** overexpression would also show improved memory. Increase of magnitude or duration of synaptic potentiation are offered as two potential explanation (Drier et al., 2002). Since only effects on memory and none on learning were observed in this study, the authors favoured the second explanation. Giving the learning improvements of ***aPKCΔ*** expression found in this study, an increase in the magnitude of synaptic potentiation seems also likely.

Since *PKC*s are evolutionary conserved, evidence for an effect on memory and learning should also be found in different species. Indeed, an *aPKCΔ* analong, *PKMζ*, was found in *Apylsia*. This constitutive active protein is formed by cleavage of PKC Apl III (Bougie et al., 2012). Chemical blocking of PKMζ leads to loss of seven-days old memory (Cai et al., 2011). Similar effect could be demonstrated in rats. Injecting a PKMζ inhibitor in the hippocampus reverses one-day old spatial memory (Pastalkova et al., 2006). Injection in the cortex abolishes long-term associative memory (Shema et al., 2007). Overexpression of *PKMζ* in the neocortex leads to an improved long-term memory (Shema et al., 2011). However, the effect of *PKMζ* for learning was questioned after two studies have shown that *PKMζ* null mice behave normally (Lee et al., 2013; Volk et al., 2013). Memory could be still chemically inhibited by ZIP. As a possible explanation, raised levels of a different *aPKC*, *PKCι/λ*, were shown in a later study (Tsokas et al., 2016). It was proposed this other *aPKC* could compensate for the missing *PKMζ*. Since *Drosophila* only has one *aPKC* no such compensation is expected. Also, no compensation of the *aPKC* knockout was observed in this study. Further, the effectiveness of pharmacological inhibitors was questioned (Wu-Zhang et al., 2012). An alternative approach would be a conditional knockout of *aPKC* in adult mice. If tools for genetic manipulations are not available for other model organisms changing to a self-learning paradigm could be helpful. Due to the conserved nature of *PKCs* it would be plausible to assume a truncated form of aPKC is also necessary in flies for memory formation and maintenance. Such protein was also found in *Drosophila* (Drier et al., 2002). Testing the flies in a memory task with *aPKC* knockout after training could give valuable insight.

It is not clear if *aPKC* and *FoxP* are interacting (Fig. 29A). Reportedly, *FoxP* downregulates targets (Li et al., 2004; Spiteri et al., 2007; Vernes et al., 2007). An increase in *FoxP* expression could downregulate *aPKC* (Fig. 29B). In the case of *FoxP* knockout *aPKC* expression would increase due to missing *FoxP*-downregulation (Fig. 29C).

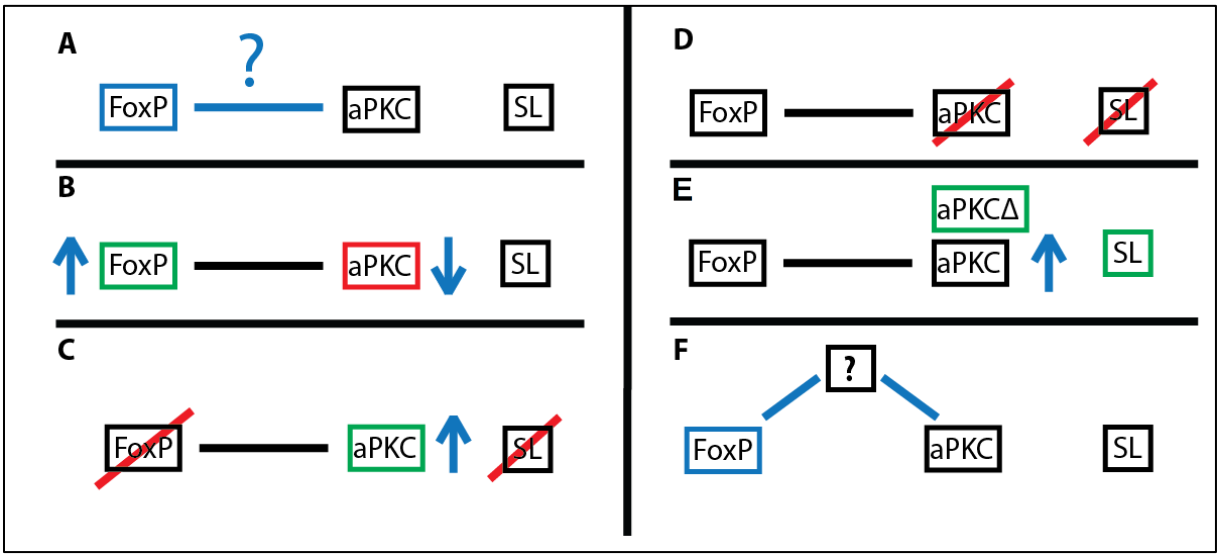

Figure 29: Schematics of possible FoxP-aPKC interactions. SL = self-learning, green box/arrow up = upregulated, red box/arrow down = downregulated, crossed out = no expression/learning

However, knockout of *aPKC* led to self-learning impairment (Fig. 29D). This is in direct contradiction to figure 29B. On the other hand, upregulation *aPKC* by expression of *aPKCΔ* improved self-learning ability (Fig. 29E). This again would contradict the assumption of figure 29C. The results indicate that *FoxP* and *aPKC* are not directly interacting, but a third intermediate gene is involved (Fig. 29F).

When looking into the literature most of the *aPKC* studies in *Drosophila* were evaluating the developmental effects of *aPKC*. Main *aPKC* functions were identified within the *hippo* (*hyp*) or the *hedgehog* (*hh*) pathway (Enderle and McNeill, 2013; Jiang et al., 2014). There, *aPKC* forms the PAR complex with *bazooka* (*baz*) and *par6* (Enderle and McNeill, 2013; Soriano et al., 2016; Thompson, 2022). Mutations in *baz* were reported as homozygous lethal, so lethality or severe impairments could be suggested after developmental knockout (Wieschaus and Noell, 1986). *baz* knockout in all neurons of the adult flies did not affect learning ability. As *baz* knockout implies non-functional PAR complex, a function of pathways involving this complex in operant self-learning could be excluded. Moreover, knockout flies even seemed to outperform control flies. However, additional experiments are necessary to make a final conclusion. Shortening the training time similar to the *aPKCΔ* experiments could be a first step. *baz* was shown to directly bind to *aPKC* (Wodarz et al., 2000). In theory, a knockout of *baz* should increase the amount of *aPKC* similar to the effect of *aPKCΔ* expression, by not binding *aPKC* within the PAR complex. Therefore, a knockout of *baz* could also lead to an improved learning ability.

So far *baz* has only been studied in *Drosophila* in a developmental context (Chen and Zhang, 2013; Thompson, 2022) and behavioural effects were not dissected. Our assumption would be that *baz* has no direct effect, but the knockout could increase the amount of available *aPKC*. This would then mimic the effect of expressing ***aPKCΔ***.

It has been proposed previously that different *PKC* isoforms are able to compensate for each other (Tsokas et al., 2016). Noteworthy, this is not the case for *aPKC* in *Drosophila*. Considering the *aPKC* knockout could not be compensated by another isoform this could suggest *aPKC* as the only relevant one for operant self-learning. Since not all *PKCs* were tested it cannot be excluded that the remaining three isoforms could also have an effect. However, this seems unlikely for isoform *inaC*, that is specifically expressed in the eyes. ***Pkcδ*** is not expressed in motor neurons and can likely be discounted (Allen et al., 2020). The remaining candidate of the *PKC* family would therefore be *PKC98e*. So far, no g-RNA line of *PKC98e* was available and working with RNAi lines has been proven to be challenging in the past (Colomb and Brembs, 2016). So, it was not possible to test the effects of a complete knockout.

Colocalization of *FoxP* and *aPKC* could be observed in the VNC. Overlap could be found in the wing motor neurons. Since flight behaviour is tested, the involvement of these neurons seems plausible. Strong *FoxP* expression can be found in the fused last abdominal segment. Since the flies are using their abdomen for steering during flight, this could be a possible explanation. No colocalization could be observed in the fly brain. This could be one explanation why the local *FoxP* knockouts in the brain did not show any effects on learning behaviour. *aPKC* is not expressed in the targeted regions with *FoxP* expression in the brain. As the results suggest, both *aPKC* and *FoxP* are involved in operant self-learning. Areas with overlap should be therefore the main focus for following research.

4.4 Blocking of brain areas

Liu and colleagues reported learning defects after blocking the PCB or EB with TeTxE (pers. communication). To verify the results the respective driver lines were retested using TeTxE and Kir2.1. Blocking with Kir2.1 did not lead to learning impairment for the four tested lines. Additionally, three of the lines were tested by crossing them with another TeTx variant, TeTxG. Here, flies showed unaffected learning performance as well. For the first three experiments with TeTxG none of the control crosses showed learning behaviour. They should not have any TeTx or Kir2.1 expression and should behave like wildtype flies. A problem with the food or the fly stock were considered. The three crosses were raised in parallel under the same conditions for each experiment. Since only one line showed this problem in three following experiments a problem with the fly stock seems to be the likely explanation.

Retesting of the lines using TeTxE revealed similar although less prominent effect as reported by Liu. Even though *GMR52B10*, *GMR64H04* and *GMR20A02* were not significantly different, the PIs were still increased. The effect was weaker than reported by the collaborating workgroup. They obtained PIs around 0, showing clear learning impairments. A possible explanation could be a difference in the power of the laser used for punishment. The intensity of the laser they used to punish the flies was not sufficient to kill them within a training period. While in this study the laser kills the animals within 15 seconds of exposure. Thus, the intensity of the punishment could have direct impact on memory formation. The learning PI could be proportional to the intensity of the laser. Thereby, high punishment intensity could overwrite weak learning impairment. It was shown in larva that the strength of learning correlates to the strength of the stimulus (Rohwedder et al., 2012; Schleyer et al., 2011). This correlation should be kept in mind for future experiments. The range of the PIs in the pretest as well as in the test periods seems very narrow for the collaborating group. Most of the flies ranging from -0.3 to 0.3 in the first two periods. The behaviour of the flies is usually very variable, ranging from around -0.8 to 0.8 in our experiments. Flies with higher or lower PIs in the pretests might have been excluded from the analysis, or the experiment could have been terminated early. No such selection did take place in this study. It is not clear if and how this is affecting the results.

TeTx is blocking chemical synapses while not affecting electrical synapses (Kitamoto, 2002; Phelan and Starich, 2001). A block with Kir2.1 on the other hand should silence all neurons regardless of the type. If electrical synapses would be the only type involved, expression of TeTx should show no effect. The expression of Kir2.1 should reliably block neurons in the targeted areas, regardless of synapses type. It is therefore surprising that Kir2.1 was not able to abolish self-learning while TeTx supposedly did. Potentially, Kir2.1 expression levels were not high enough to fully block neuronal activity. Similar finding was observed in a gustatory study (Jaeger et al., 2018). Here only the expression of TeTx appeared to block the gustatory neuron IR94e. The block with Kir2.1 had no effect.

It is noteworthy that *FoxP* is not expressed in the EB. If this region would have an effect for operant self-learning, it would indicate the existence of a different, *FoxP* independent, mechanism. Also, *aPKC* does not seem to be expressed in the EB. The lower laser intensities and narrow PI distribution seem to be the two main differences in the two studies. Future experiments will be necessary to determine the impact. Ideally the experiments should be replicated by a third lab (Kortzfleisch et al., 2022).

4.5 Outlook

This study provided first attempts to further investigate the involvement of *FoxP* in operant self-learning. The missing brain areas vest and saddle should be investigated. It would be necessary to find Gal4 lines with matching expression patterns to *FoxP*. So far, the data suggest that the main area of interest is the VCN. Here, it is necessary to identify the precise neurons, where *aPKC* and *FoxP* are overlapping. These areas should then become focus of additional research.

Studying the effects of *aPKC* on memory formation and maintenance could also be interesting. The results from different studies across species indicate the involvement of *aPKC*. Here a classical odour learning task could give valuable insight.

Blocking the PCB or the EB led to much weaker effects than reported from the other work group. Further studies will be needed to find the reason for the difference to make the findings reproducible. Repetition of the experiments with reduced laser intensity could be a first step.

5. Summary

In this study we show that the knockout of *FoxP* in all neurons had no immediate detrimental effect on the learning ability of *Drosophila.* It might be important for maintain the learning capacity in aging flies. Flies were not able to learn anymore 14 days after the knockout. No brain region could be determined, where *FoxP* expression is necessary for operant self-learning. *FoxP* expression is not needed in the PCB, the nodulli, the FB or the Ato-cluster for operant self-learning.

aPKC was shown to be important for the self-learning ability of the flies. *PKC53e* does not seem to be involved. A knockout of *aPKC* in all motor neurons led to learning impairments. The same effect could be observed by limiting the expression to *FoxP-iB* positive neurons. We found strong indication for an interaction between *aPKC* and FoxP. But there is most likely not a direct interaction. Overexpression of *aPKC* led to improvement of the learning ability of flies.

FoxP does not seem to be important in the brain itself for operant self-learning. The important area seems to be rather the VCN. Here, colocalization of *FoxP* and *aPKC* could be observed. No colocalization was found in the brain. The results suggest that *FoxP* and *aPKC* both are required for operant self-learning. So, areas with *FoxP/aPKC* colocalization could be the relevant ones.

Further, the correct formation of the PAR-complex seems not important for operant self-learning.

Lasty, it was not possible to reproduce the strong learning impairments reported when blocking the PCB or the EB with Kir2.1. Learning impairments could be observed when TeTxE was utilized.

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7. Supplement

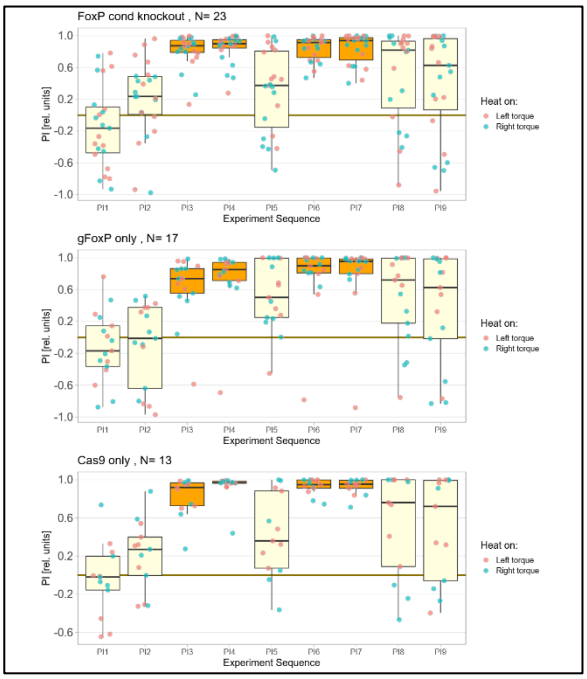

Figure S1: Conditional FoxP knockout in adult flies, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52948

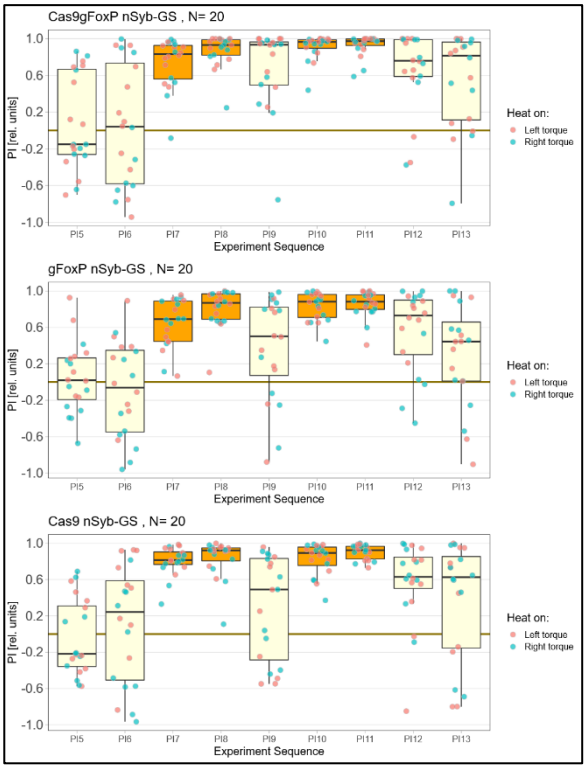

Figure S2: Conditional FoxP knockout in adult flies, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52963

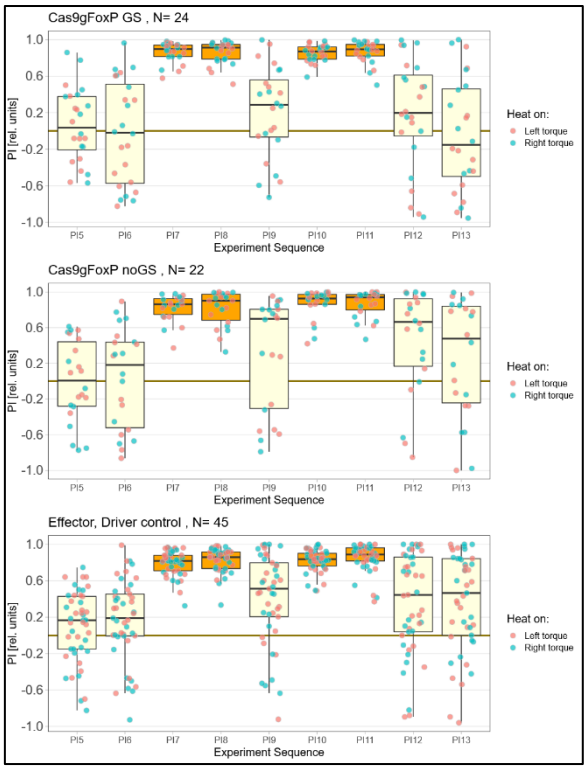

Figure S3: Testing of 14-day old flies with adult FoxP knockout, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52964

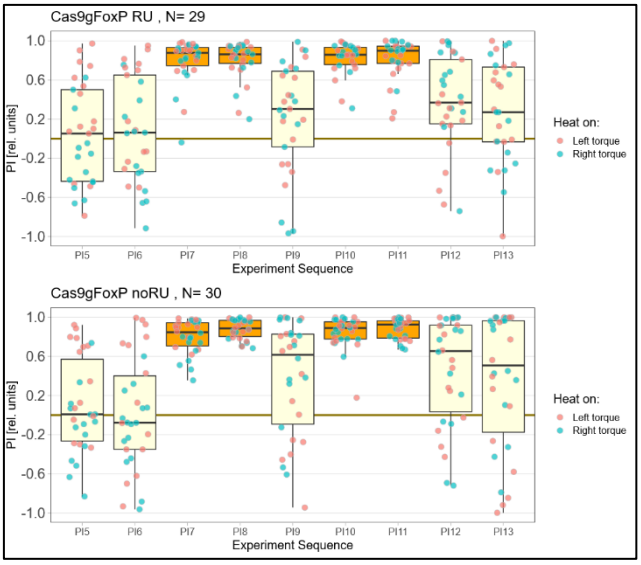

Figure S4:Testing of 7-day old flies after FoxP knockout, experimental sequence,, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52965

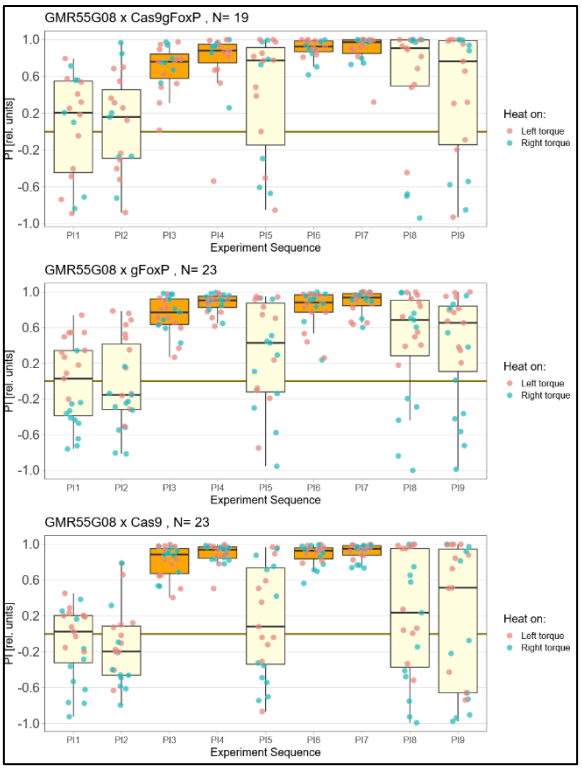

Figure S5: Local knockout of FoxP in the PCB, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52953

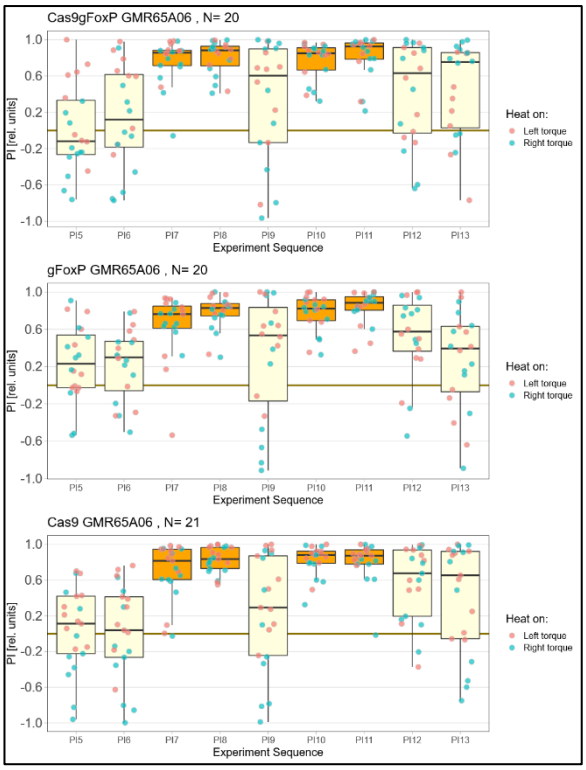

Figure S6: Local FoxP knockout in the PCB, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52956

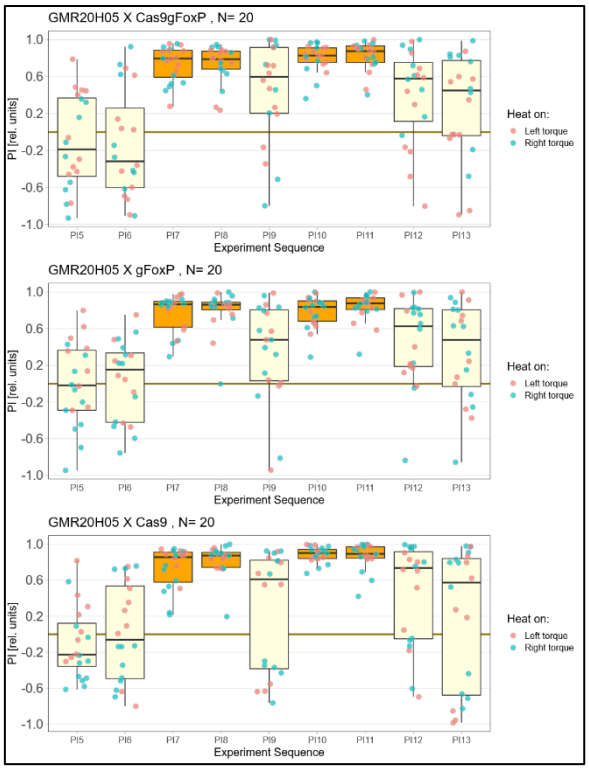

Figure S7: Local FoxP knockout in the PCB, FB and noduli, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52951

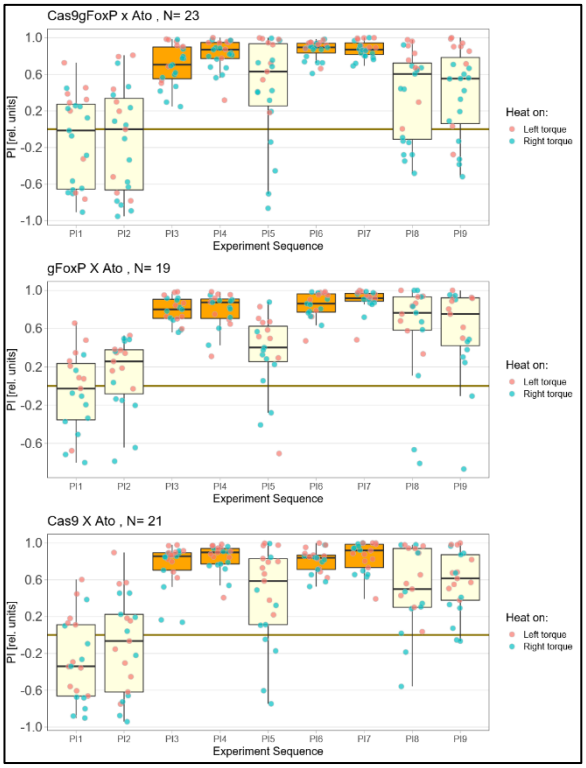

Figure S8: Local FoxP knockout in the Ato-cluster, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52946

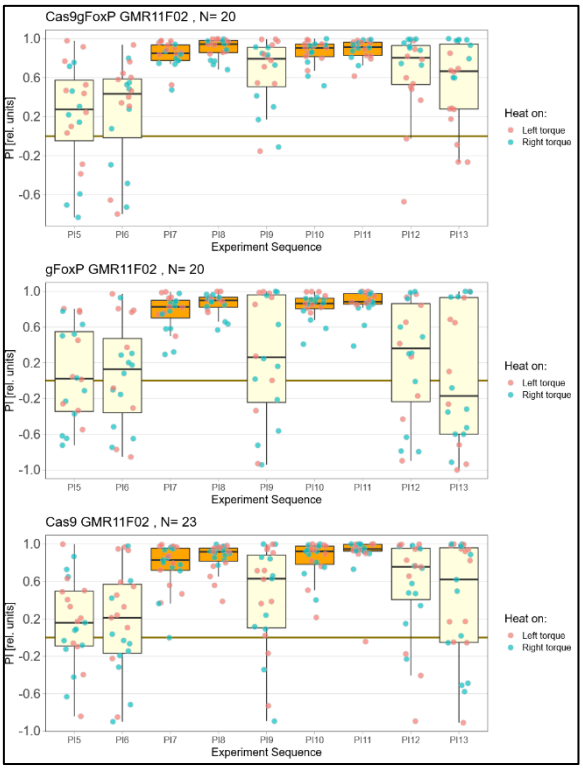

Figure S9: Local FoxP knockout in the expression area of GMR11F02 (no coexpression), experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52949

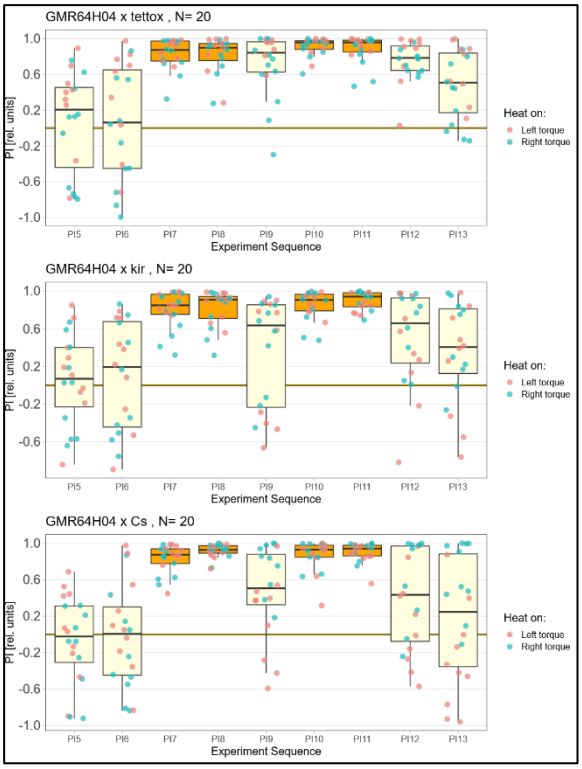

Figure S10: Blocking of the EB with TeTxG or KIR2.1, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52956

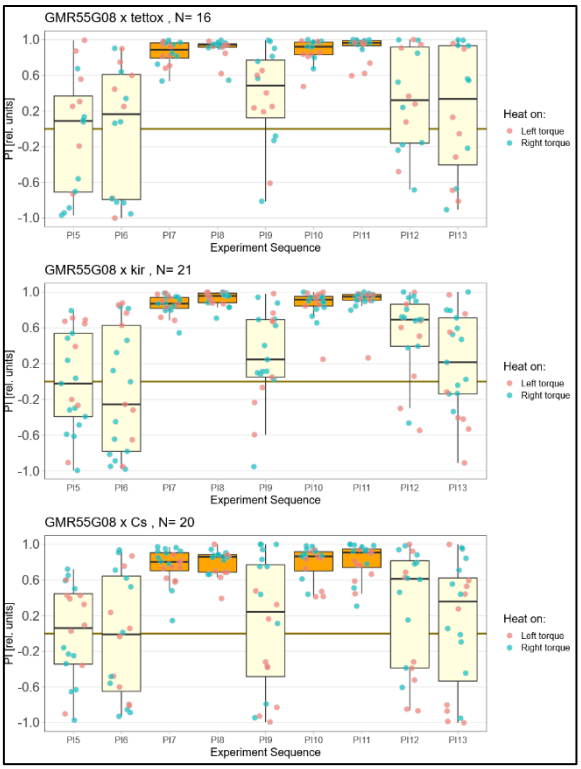

Figure S11: Blocking of the EB with TeTxG or Kir2.1, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52954

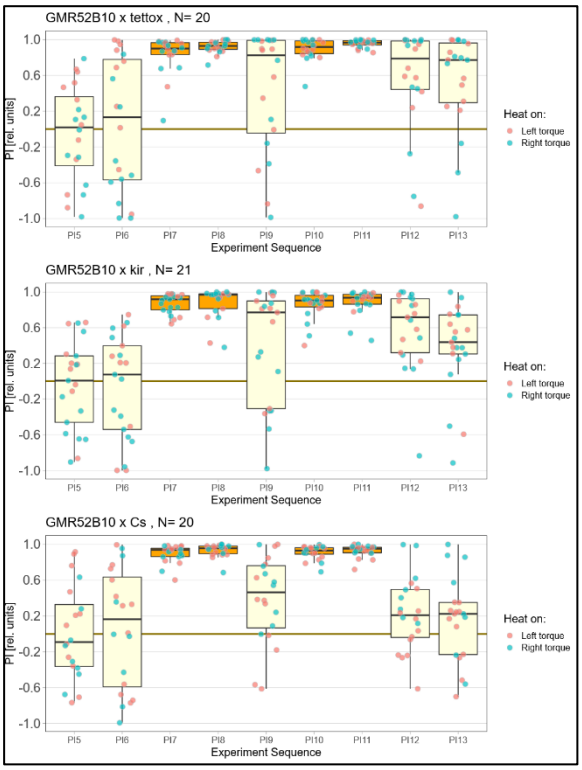

Figure S12: Blocking of the PCB with TeTxG or Kir2.1, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52952

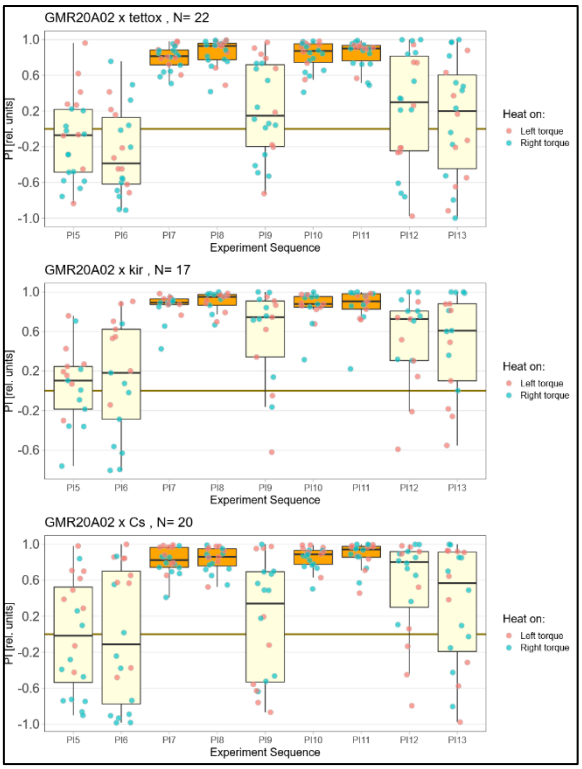

Figure S13: Blocking of the PCB with TeTxE or Kir2.1, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52950

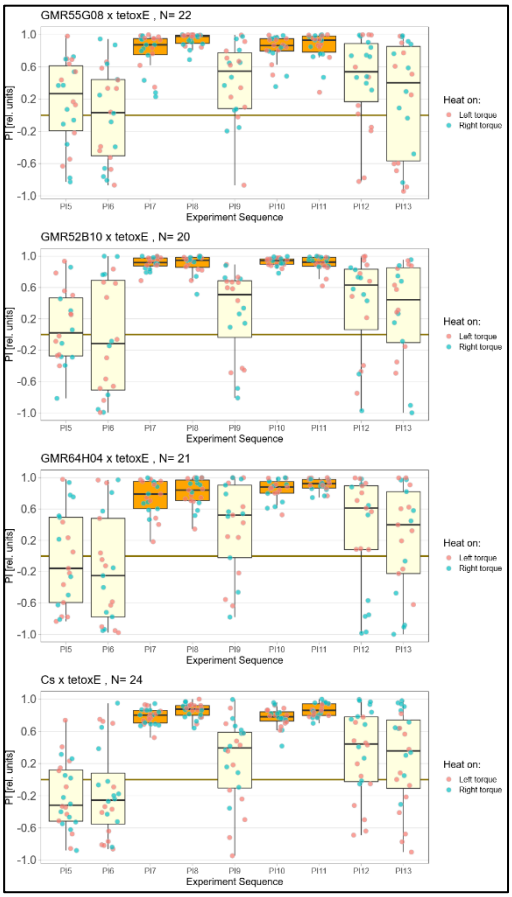

Figure S14: Retesting of the previous three lines, Blocking with TeTxE or Kir2.1, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52966

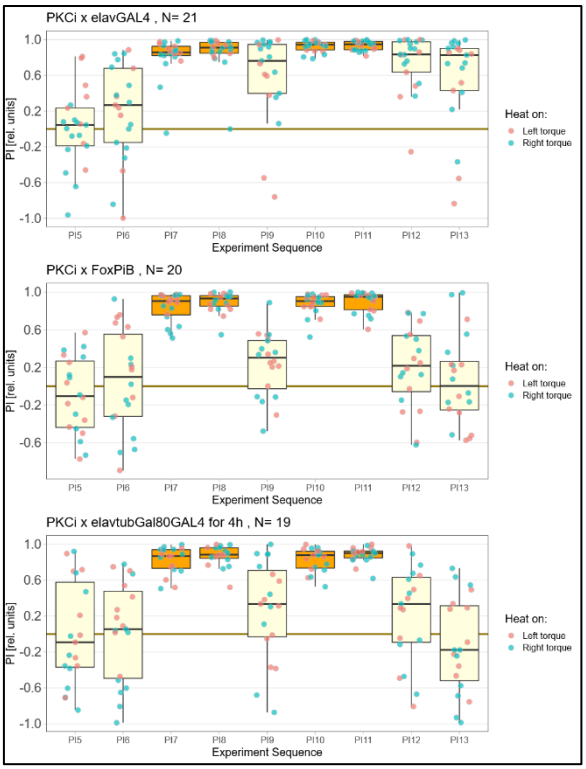

Figure S15: Expression of PKCi in all neurons during development or in adult flies, or in FoxP-iB neurons, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52958

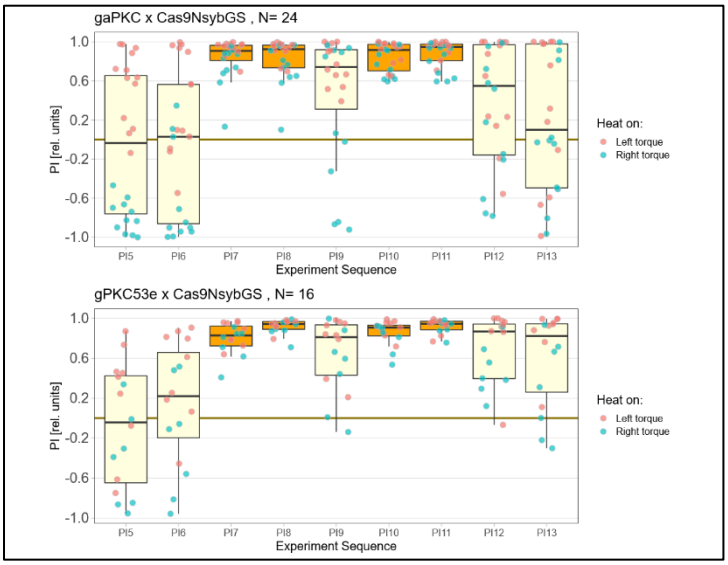

Figure S16: Knockout of aPKC or PKC53e in all neurons in the adult fly, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52957

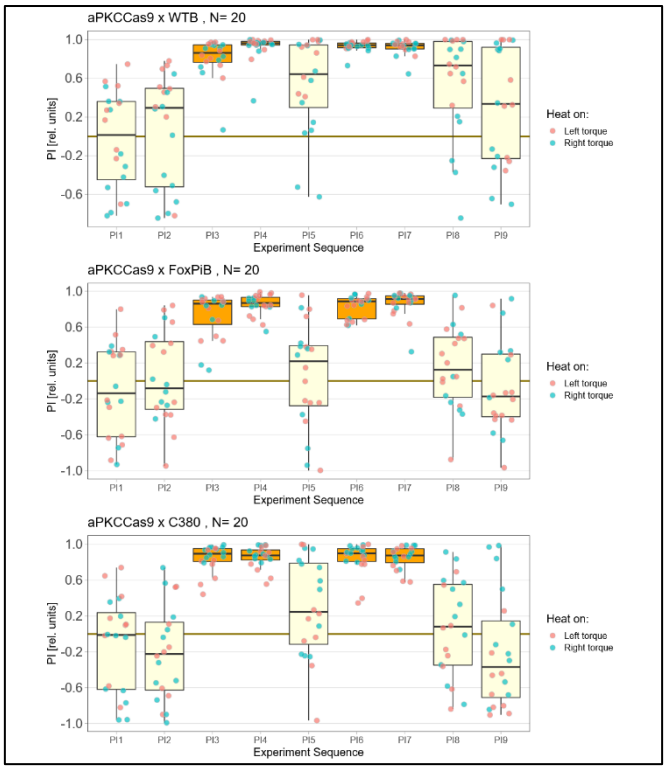

Figure S17: Knockout of aPKC in all motor neurons or FoxP-iB positive neurons, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52944

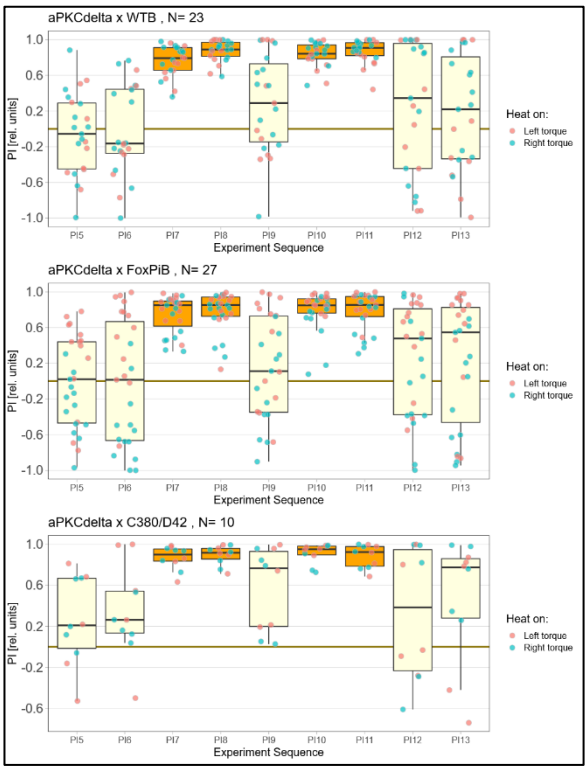

Figure S18: Expression of aPKCΔ in FoxP-iB positive or motor neurons, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52961

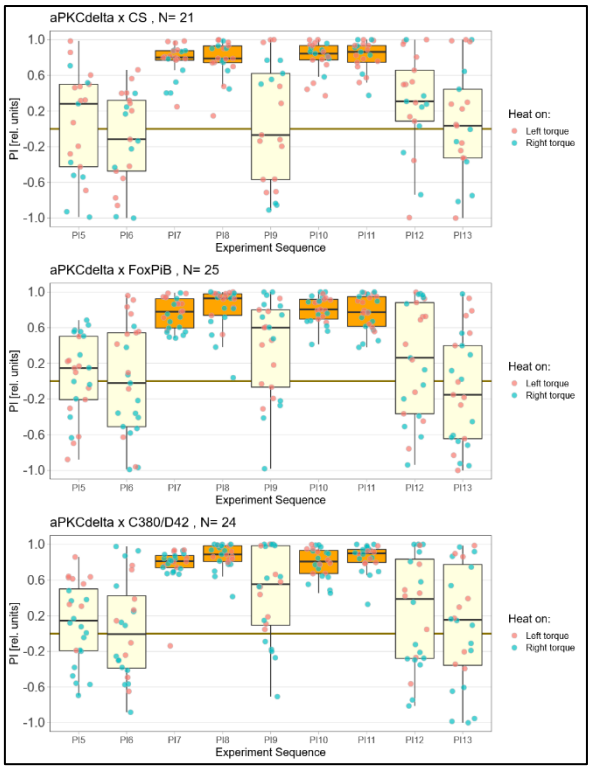

Figure S19: Expression of aPKCΔ in FoxP-iB positive or motor neurons, half the period duration (1 min), experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52962

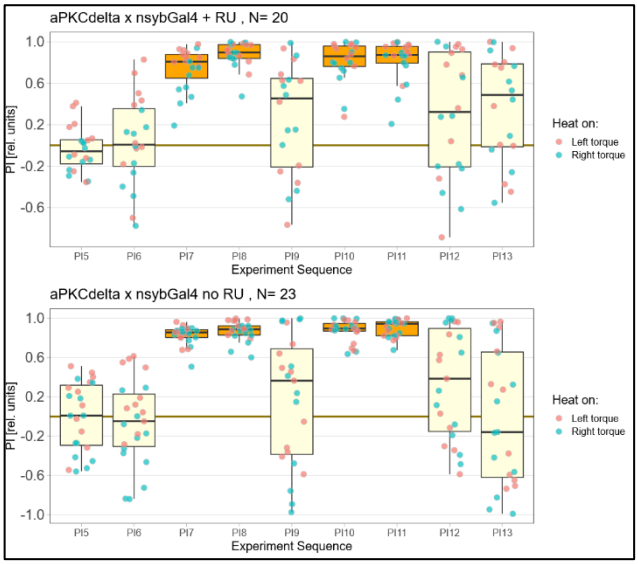

Figure S20: Expression of aPKCΔ in FoxP-iB positive or motor neurons, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52959

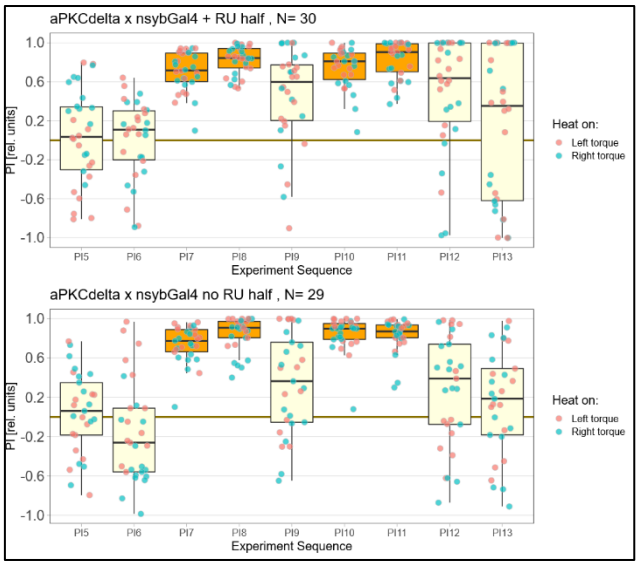

Figure S21: Expression of aPKCΔ in all neurons in adult flies, half the period length (1 min), experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52960

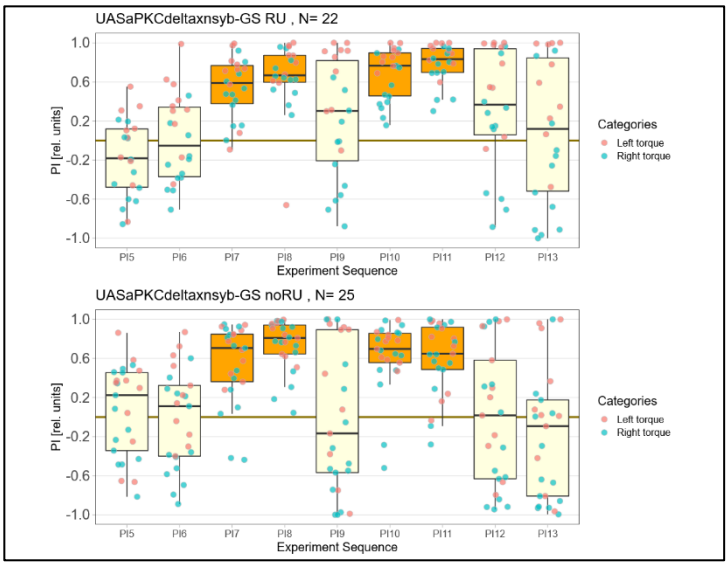

Figure S22: Retest by Amelie Hauser: expression of aPKCΔ in all neurons in adult flies, half the period length (1 min), experimental sequence, each bar representing two-minute periods, orange bars indicate training periods.

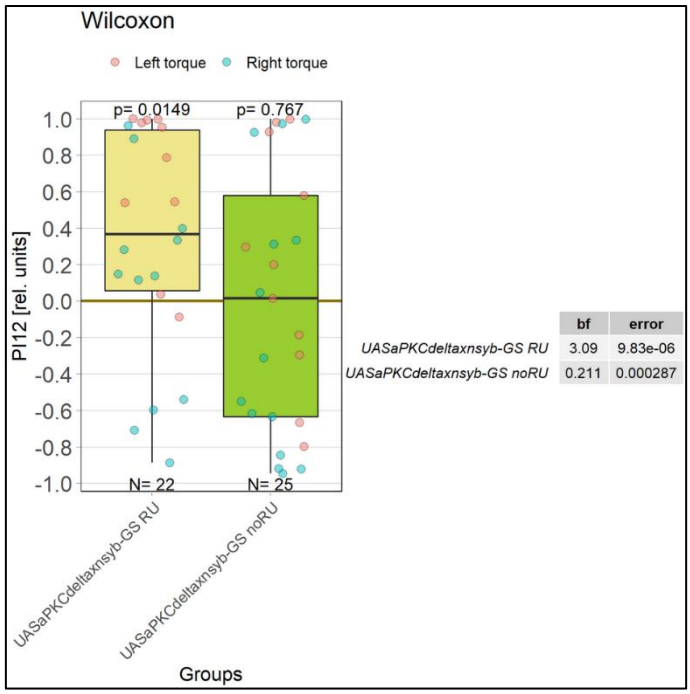

Figure S23: Retest by Ameilie Hauser, expression of aPKCΔ in all neurons in adult flies, half the period length (1 min), performance index (PI) of the first test period after the last training. Y-axis: PI of period 12, x-axis: tested groups: nsybGs-Gal4>UAS-aPKCΔ

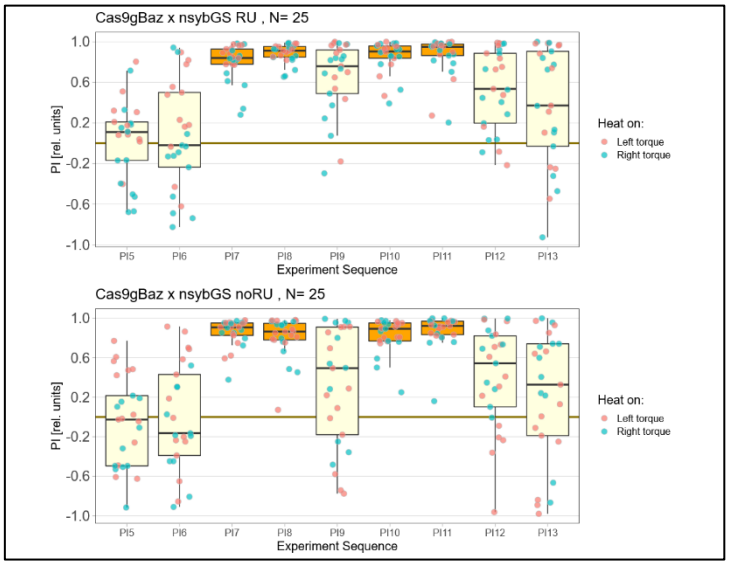

Figure S24: Knockout of BAZ in all neurons of the adult fly, experimental sequence, each bar representing two-minute periods, orange bars indicate training periods. Complete data: http://doi.org/10.5283/epub.52947

8. List of figures

9. List of tables

10. Acknowledgements

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(1) Ich erkläre hiermit an Eides statt, dass ich die vorliegende Arbeit ohne unzulässige Hilfe Dritter und ohne Benutzung anderer als der angegebenen Hilfsmittel angefertigt habe; die aus anderen Quellen direkt oder indirekt übernommenen Daten und Konzepte sind unter Angabe des Literaturzitats gekennzeichnet.

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Andreas Ehweiner