The Drosophila black enigma: The molecular and behavioural characterization of the black\textsuperscript{l} mutant allele

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Abstract

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

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1. Introduction

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

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

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

2. Materials and methods

2.1. Fly stocks and crosses

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

2.2. Molecular biology

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

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

2.3. Protein analysis

Western analysis was performed using reduced protein extracts separated on SDS PAGE gels and transferred to nitrocellulose using a semi-dry system. All filters were stained with Ponceau stain and scanned before being blocked overnight in 5% skim milk powder in 1XTBS 0.05% NP-40. Primary and secondary antibodies were applied to the filter in blocking solution. The primary antibody used was an affinity purified anti-GAD peptide antibody raised in rabbits. The commercially produced peptide, residues 138–157, was obtained from Chiron and conjugated to pertussin toxin before injection into rabbits. Cross-reacting bands were identified using HRP conjugated antibodies. Sequencing of DNA products was performed by AGRF (Australian Genome Research Facility) by gel separation of Dye-terminator reactions (Big Dye terminator RR mix, Applied Biosystems).

2.4. Enzyme assays

The enzymes assays were conducted as previously described (Phillips et al., 1993). The H^2-glutamate and H^1-aspartate (22 Ci/mm) were obtained from Amersham. All assays were linear up to 150 μg of added protein and measurable activity was lost on heating the extract. Glutamate conversion to GABA was linear up to 45 min under all assay conditions. A 30 min incubation was used for all GAD assays shown. Aspartate conversion to β-alanine
was linear up to 10 min, and plateaued at later times. A 5 min incubation was used for all AAD assays.

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

2.5. Electrophysiology

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

2.6. Buridan experiments

The method used for Buridan’s paradigm (Götz, 1980), is similar to that described in Strauss and Pichler (1998).

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

Fig. 1. Aspartate decarboxylase (AAD) activity in the black¹ mutant. A: (i) Reduced AAD activity, relative to wild-type, in protein extracts from black¹ mutant heads is demonstrated using an assay with 10 μM aspartate (using ³H aspartate 200,000 cpm), and 100 μg protein. The protein extracts were prepared in the absence of PMSF (see D). The data shows conversion of ³H aspartate to ³H β-alanine in a 5 min incubation at 37 °C. The reduced activity is significantly different from wild-type (Student’s two-tailed t-test p = 0.0028, n = 5). (ii) Enzyme assay as for A(i) but using 10 mM aspartate as substrate. The activity of the black¹ mutant was by Student’s two-tailed t-test, for two sets of data with different variance, or similar variance, as appropriate.

B: AAD activity in protein extracts from Oregon-R flies using the assay conditions described for A(i), but with alterations to buffer pH. For assays with pH below 5.8, 50 mM K⁺ acetate was used as buffer, between pH 5.8 and pH 7.6 the buffer was 50 mM K⁺ phosphate. The graph shows mean value and range for each point (n = 3). Where no error bars are shown they fall within the symbol indicating the mean value. C: Glutamate decarboxylase activity (mean±SEM) in protein extracts from black¹ flies relative to wild-type extracts. (i) Using 10 μM glutamate as substrate and the protein extracts used to analyse AAD activity in A, there was no significant difference in GAD activity (Student’s two-tailed t-test, p = 0.37, n = 5). (ii) In experiments using 10 mM glutamate, GAD activity in mutant and wild-type is identical, as observed previously (Student’s two-tailed t-test, p = 0.88, n = 3). D: Conversion of aspartate to β-alanine in the presence and absence of the serine protease inhibitor phenyl-methyl-sulfonyl fluoride (PMSF). Conversion is decreased if PMSF is added to the homogenisation buffer (p < 0.015 for both OR-R and black AAD assays, Student’s two-tailed t-test, n = 3). Addition of PMSF immediately prior to assay also results in decreased conversion of aspartate to β-alanine. The ratio of black¹ to wild-type activity is not affected.
reconstructed from the stored sequence of velocity vectors that represent direction and speed between consecutively recorded positions. All of the data shown was extracted from these recordings (Strauss and Pichler, 1998). The angle of orientation between fly and approached target was measured every 0.2 s (1500 recordings per fly). Data points with angles between 0° and 5° as well as −5° and 0°, between 5° and 10° as well as −10° and −5°, etc., were pooled and their normalized frequency plotted. The curve for random orientation was calculated as described in Strauss and Pichler (1998).

3. Results

3.1. Aspartate decarboxylase activity in black1 flies

We had developed an assay for acidic amino-acid-decarboxylase activity (Phillips et al., 1993; Featherstone et al., 2000). In studies of flies heterozygous for deletions of the Dgad2 locus and a black mutation we had not found any statistically significant reductions in glutamate/aspartate decarboxylation (Phillips et al., 1993). As genetic data and genomic sequence were consistent with the black
and DGad2 loci being synonymous, we decided to assay aspartate decarboxylase activity in homozygous black \(^1\) flies. Although separation of active GAD enzymes has been achieved by immunoprecipitation, our antibodies would not precipitate the enzyme, so we evaluated DGAD2 activity using crude protein extracts. The \(K_M\) for Drosophila glutamate decarboxylase had been established as 11 mM using partially purified enzyme (Chude et al., 1979). Altering the aspartate concentration in our assay from 10 mM to 10 \(\mu\)M revealed a significant reduction in aspartate conversion to \(\beta\)-alanine in black \(^1\) head protein extracts relative to wild-type (Fig. 1A(i) and (ii)) (PMSF was omitted from the buffer, see Fig. 1D). This is the first demonstration of a significant decrease in aspartate decarboxylase activity in a black mutant, and is consistent with DGAD1 and DGAD2 decarboxylases differing in their substrate specificity. We then used 10 \(\mu\)M aspartate as substrate in studies of pH effects on activity. In the studies of Chude et al. (1979), assays of DGAD activity using the crude enzyme showed two pH optima (at pH 5.0 and pH 7.2) while the partially purified DGAD enzyme had a single peak of activity at pH 7.2. Using 10 \(\mu\)M aspartate and crude protein extracts from heads of Oregon-R wild-type flies, two peaks of AAD activity were seen, the first peak at pH 5.4 and a second peak at pH 6.8 (Fig. 1B). At pH > 8 the assays could not be evaluated as there was non-enzymatic conversion of the substrate (data not shown). All subsequent AAD assays were conducted at pH 7.0, where there was a measurable difference between black \(^1\) and Oregon-R (Fig. 1A(i)) and where any small change in pH allowed reproducibility between assays. Protein extracts from heads of black \(^1\) mutant homozygotes showed a significant reduction in activity in multiple experiments using 10 \(\mu\)M aspartate at pH 7.0. This reduction in enzyme activity in black \(^1\) mutants is seen when aspartate, but not glutamate, is used as a substrate (Fig. 1C(i) and (ii)).

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

### 3.2. The Dgad2 transcripts

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

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**Fig. 3. Effect of the mutations in black \(^1\) on the encoded protein.** (A) Alignment of the active site of black/DGAD2 from D. melanogaster, D. pseudoobscura and A. gambiae and of the related PLP-dependent decarboxylases, D. melanogaster DGAD1 (Jackson et al., 1990), Feline Gad67 (Kobayashi et al., 1987) and Human Gad65 (Bu et al., 1992). The asterisk identifies the conserved tyrosine, residue 347, that is altered to histidine in the black \(^1\) mutant. (B) Diagrammatic representation of the two wild-type proteins encoded by the black/Dgad2 locus if both translation start sites are used, bars 1 and 2. The changes in these proteins resulting from the black \(^1\) frame-shift mutation at 530 bp, bars 3 and 4.
two transcripts. We considered the possibility that the two transcripts might be differentially expressed, one in the cuticle and the other in glial cells. By analogy, in a gene encoding an evolutionarily related PLP decarboxylase, \textit{dopa-decarboxylase (ddc)}, different transcripts are used for neuron-specific and cuticular expression (Scholnick et al., 1986). Mutations affecting DGAD2 expression in the cuticle but not the nervous system could be a possible explanation for the difference in the visual phenotype between \textit{ebony} and \textit{black}.

The genomic sequence was analysed for potential transcription start-sites and two with equal likelihood (0.99) were predicted by the program (Reese, 2001). The first site is at –143 bp, and the second at +77 bp from the first putative translation start codon in the cDNA. The second is therefore between the two translation start codons (Fig. 2A and B). Two similar sites are predicted in \textit{D. pseudoobscura} (Fig. 2A) and both of the putative translational start sites are also retained. RT-PCR and sequencing confirmed that a transcript extending from the putative first start site (A1 in Fig. 2B) is present in adult head and in adult bodies (Fig. 2C). This is in agreement with the original cDNA (Phillips et al., 1993) that extends 5’ to the A3 start site (Fig. 2B). However using a more 3’ primer (primer 2 in Fig. 2B) RT-PCR with both body and larval RNA produced a strong reaction product (Fig. 2C) suggesting that there may indeed be a shorter transcript produced. Using primer extension a product was identified in adult head RNA corresponding to initiation at A1. A similarly sized product was observed using larval RNA (Fig. 2D–primer 1). The longer transcript must therefore be present in head, body and larvae. An extension product consistent with a shorter transcript may be present in head mRNA, however the signal is weak, and the transcript is shorter than that expected from initiation at A2 (Fig. 2D–primer 2). While proving that the longer transcript is produced, the data does not confirm that tissue-specific differences in transcription of the \textit{black} gene are present, nor that it might account for the \textit{black} mutant phenotype.

### 3.3. Molecular analysis of the Dgad2 gene

The \textit{black1} mutation arose spontaneously (see Lindsley and Zimm, 1992) and is homozygous viable. For these reasons the molecular defect is likely to be confined to the \textit{black} locus. Sequencing of the \textit{black1} promoter revealed many single base changes, but none affecting the transcriptional start sites. The open reading frame encoding DGAD2 was then sequenced in the \textit{black1} mutant using overlapping PCR products derived from four individual preparations of genomic DNA. In \textit{black1} the replacement of four bases (ATCC) by an eight base pair insertion (TACCTACC) at position +530 bp in the cDNA sequence results in a frame-shift. If expressed this would produce a truncated, enzymatically-inactive protein (Fig. 3B). There were also 18 single base pair substitutions in the \textit{black1} mutant sequence, compared to the cDNA sequence (Phillips et al., 1993). Of these, only one a T to C substitution at +1042 bp, resulting in a tyrosine (Y) to histidine (H) alteration at residue 348, might be functionally significant as this Y is conserved across species and in related decarboxylases (Fig. 3A). The \textit{Dgad2} cDNA clone, the G-2 genomic clone (Phillips et al., 1993), both derived from Canton-S, and the genomic sequence in Flybase (Scaffold No AE003641, Ashburner et al., 1999; Adams et al., 2000; Celniker et al., 2002) were identical in sequence. Laboratory strains, Oregon-R and \textit{w1118}, also showed conservation of the \textit{Dgad2} sequence. Overall, the amino acid sequence in all

![Fig. 4. The black/DGAD2 protein is absent from \textit{black1}. A: Western blots showing a protein at 58 kDa in the heads of adult wild-type flies that is not observed in the \textit{black1} mutant. The Ponceau stained panel is of the portion of the antibody probed filter, showing even loading. B: (i) Western blot of probed proteins from OR-R and \textit{black1} bodies. The data from this blot, are consistent with a 58 kDa protein being absent from \textit{black1} flies. This is confirmed in (ii) which shows the densitometric traces from the last two lanes superimposed. There is a major peak of cross reacting material present in wild-type that is missing from \textit{black1}. Other cross-reacting species are common to both genotypes.](image-url)
strains studied, with the exception of black, shows a high level of conservation (>99% identity).

3.4. DGAD2 protein in black

Soluble proteins extracted from the heads or bodies of wild-type adult flies and black mutant homozygotes, were compared on Western blots. When probed with affinity purified anti-DGAD2 antibodies, a protein around 58 kDa is identified in wild-type but not black mutant extracts (Fig. 4A and B(i)). Densitometry confirmed that this protein species was missing from black and that the slightly lower molecular weight protein seen in black extracts was present, but masked by the DGAD2 signal in wild-type extracts (Fig. 4B(ii)). The first in-frame AUG in the original Dgd2 cDNA would be expected to produce a protein of 64 kDa. The second in-frame AUG would produce a 58 kDa protein. There is no evidence on the Western blots for the larger 64 kDa protein, and extracts of heads and bodies run on the same gel show coincident mobility of the antibody reacting proteins (data not shown). No equivalent protein is produced in black mutant flies.

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

3.5. Physiological assays and phenotypes

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

The expression of Dgd2 in the first optic ganglion suggests a neuronal/visual system role for black (Phillips et al., 1993). However black mutant flies have normal ERGs, consistent with the published literature (Hotta and Benzer, 1969; Fig. 6). Extensive studies comparing black under both dark-adapted and ambient light conditions, and at different light intensities showed no differences from wild-type. This included black flies that had been outcrossed to remove any modifiers. We generated the double mutants black;tan, black;tan, black;ebony and ebony;tan. The pigmentation of the double mutant flies was consistent with that expected from the published literature. However, none of the double mutants had on- or off-transients (for example see Fig. 6). The data indicate a functional difference between the ability to produce β-alanyl-histamine.
flies. The ebony flies show no transient responses at light-on or light-off indicating a failure of transmission from the photoreceptor cells to those in the first optic ganglion. In contrast the black flies show no effect on the transient responses. The phenotype of the black, ebony double mutant is identical to that of ebony flies.

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

3.6. Aberrant orientation behaviour in black flies

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

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

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

Fig. 7. Analysis of Buridan’s paradigm traces. (A) Examples of traces of single flies walking for 5 min between inaccessible visual landmarks (symbolized by solid bars flanking the circles). The genotypes are as indicated. The traces for ebony and wild-type Berlin were generated in a separate series of experiments. (n = 11 for ebony and black, 20 for wild-type Berlin, 16 for Oregon-R and 14 for Canton-S). (B) Mean±SEM of the total distance walked during the 5 min experiments for each genotype. The black data are not significantly different from Oregon-R or Canton-S (see text). (C) Mean walking speed±SEM. Mutants do not differ significantly from the wild-types (see text). (D) Mean±SEM of the number of transitions between the landmarks (number of walks) for each genotype. Both ebony and black differ significantly from the wild-types. See text for probabilities. (E) Mean±SEM of error angles that mark the 50% of walks that deviate the least from the target. The dotted line indicates the 50% error angle for random walking. The mutants differ significantly from the wild-types. For black vs. Oregon-R p > 0.001. (F) The angle of orientation between fly and approached target was measured every 0.2 s (1500 recordings per fly). Data points with angles between 0° and 5° as well as −5° and 0°, between 5° and 10° as well as −10° and −5°, etc., were pooled and their normalized frequency plotted. The curve for random orientation was calculated as described (Strauss and Pichler, 1998). The data for black falls between the random curve and the curves for the two wild-type strains, Oregon-R and Canton-S.
F, the area underneath the frequency curve in Fig. 7F is bisected at this value. The mean frequency distribution of the mutant black flies showed a broad plateau between 0° and 40° instead of an upward trend towards 0° error angle in the wild-type strains (Fig. 7F). Their fixation abilities were nevertheless clearly better than random, but significantly worse than either Oregon-R or Canton-S ($p < 0.001$; Fig. 7F). Both Oregon-R ($p = 0.0298$) and Canton-S ($p = 0.0016$) also differ from black in initiating walks (Fig. 7D). If fixation is the impetus driving the initiation of walks, then
the reduction in the number of walks initiated by black flies may be a reflection of the inability of black to fixate effectively.

4. Discussion

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

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

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

The putative GAD2 homologues in D. pseudoobscura and A. gambiae show considerable sequence identity to the D. melanogaster gene with D. pseudoobscura GAD2 having 80% identity and 97% similarity to the D. melanogaster protein. In A. gambiae identity is around 70% for sequence that is annotated although the initiating methionine and adjacent amino terminal sequences could not be identified. This conservation across dipteran species suggests that mutations affecting protein function would be detrimental. Sequencing of homozygous black1 mutants revealed that black1 is functionally a null for the encoded aspartate decarboxylase. At least two mutations are functionally significant, the tyrosine to histidine in a domain likely to be important in substrate recognition, and an insertion/inversion mutation resulting in a frame shift. The structural mutation resembles a transposable element footprint and although both these mutations have occurred spontaneously, it is not possible to determine which mutation was the primary event. A large number of silent changes present in the mutant may reflect the genetic background of the parental strain, or result from an accumulation of mutations in the unselected gene.

The apparently normal visual phenotype of black mutants has been difficult to understand given current hypotheses. β-alanine can be conjugated to histamine and the inability of ebony flies to form carcinine has been suggested to result in their abnormal visual function and lack of ERG transients. However black flies have normal ERGs. The black mutant flies cannot make β-alanine via the decarboxylation of aspartate and hence, like ebony, should be defective in carcinine production (Borycz et al., 2002). This then poses a paradox. The absence of β-alanine in the cuticular melanization pathway creates a black fly, as does the enzymatic defect in ebony. However this similarity between black and ebony does not extrapolate to the ERG transients despite both products being expressed in the lamina. Borycz et al. (2002) have found that histamine levels are low in both ebony and black and both are deficient in carcinine. Studies by others (McDonald and Rosbash, 2001; Richardt et al., 2003) show that while black mRNA cycles in response to light/dark cues, as does histidine decarboxylase (hdc) and ebony, the black message peaks some 6–7 h earlier than the other messages i.e. black is most highly expressed in the night and hdc and ebony at dawn. Mutations in the tan gene produce abnormal ERG transients, but tan has not been cloned, and defects in vesicle cycling, and visual system changes due to abnormal development (Neckameyer et al., 2001) may be as important as any postulated enzymatic activity in determining the ERG phenotype in tan mutants. A build up of free β-alanine in ebony flies, with a consequent inhibitory effect on the lamina response, is one possible explanation for the ERG differences between black and ebony. However this phenotype would be suppressed in a black mutant where β-alanine cannot be synthesised. We observe no suppression of the ebony ERG defect in the black/ebony double mutant. Overall, the evidence for a similar bio-genic amine pathway acting in both the visual system and in the cuticle of flies is not compelling.
Out-crossing the black\textsuperscript{1} flies, and chromosomal replacement, has eliminated any unlinked modifiers, unless such modifiers are common in laboratory strains. Compensatory up-regulation of either another decarboxylase or of the uracil metabolic pathway has been considered. However, from published data (Borycz et al., 2002) on carmine levels in black\textsuperscript{1} flies, there is no evidence of an alternative source of \(\beta\)-alanine in the black\textsuperscript{1} visual system.

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

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

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

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