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The Drosophila *black* enigma: The molecular and behavioural characterization of the *black*[1] 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*[1] mutants of *Drosophila melanogaster* in order to resolve the apparent paradox of the *black* phenotype. Using *black*[1] mutant flies we show that (1) aspartate decarboxylase activity is significantly reduced in adults and at puparium formation, consistent with defects in cuticular and non-cuticular processes, (2) that the *black*[1] mutation is a frameshift, and *black*[1] flies are nulls for the black/DGAD2 protein, and (3) that behavioural experiments using Buridan's paradigm, demonstrate that *black* responds abnormally to visual cues. No ERG, or target recognition defects can be demonstrated suggesting a problem with higher order visual functions in *black* mutants.

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

1. Introduction

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

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

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

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

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

2. Materials and methods

2.1. Fly stocks and crosses

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

2.2. Molecular biology

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

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

2.3. Protein analysis

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

2.4. Enzyme assays

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

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

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

2.5. Electrophysiology

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

2.6. Buridan experiments

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

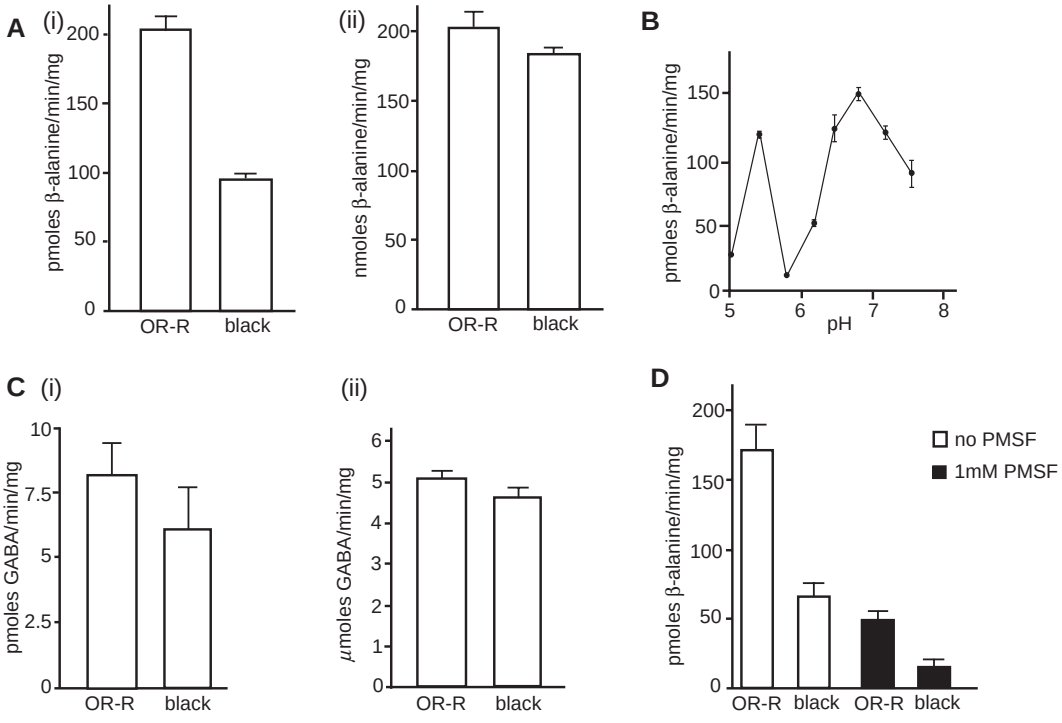


Fig. 1. Aspartate decarboxylase (AAD) activity in the *black*^{1} mutant. A: (i) Reduced AAD activity, relative to wild-type, in protein extracts from *black*^{1} mutant heads is demonstrated using an assay with 10 μM aspartate (using ^{3}H aspartate 200,000 cpm), and 100 μg protein. The protein extracts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

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

3. Results

3.1. Aspartate decarboxylase activity in black[1] flies

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

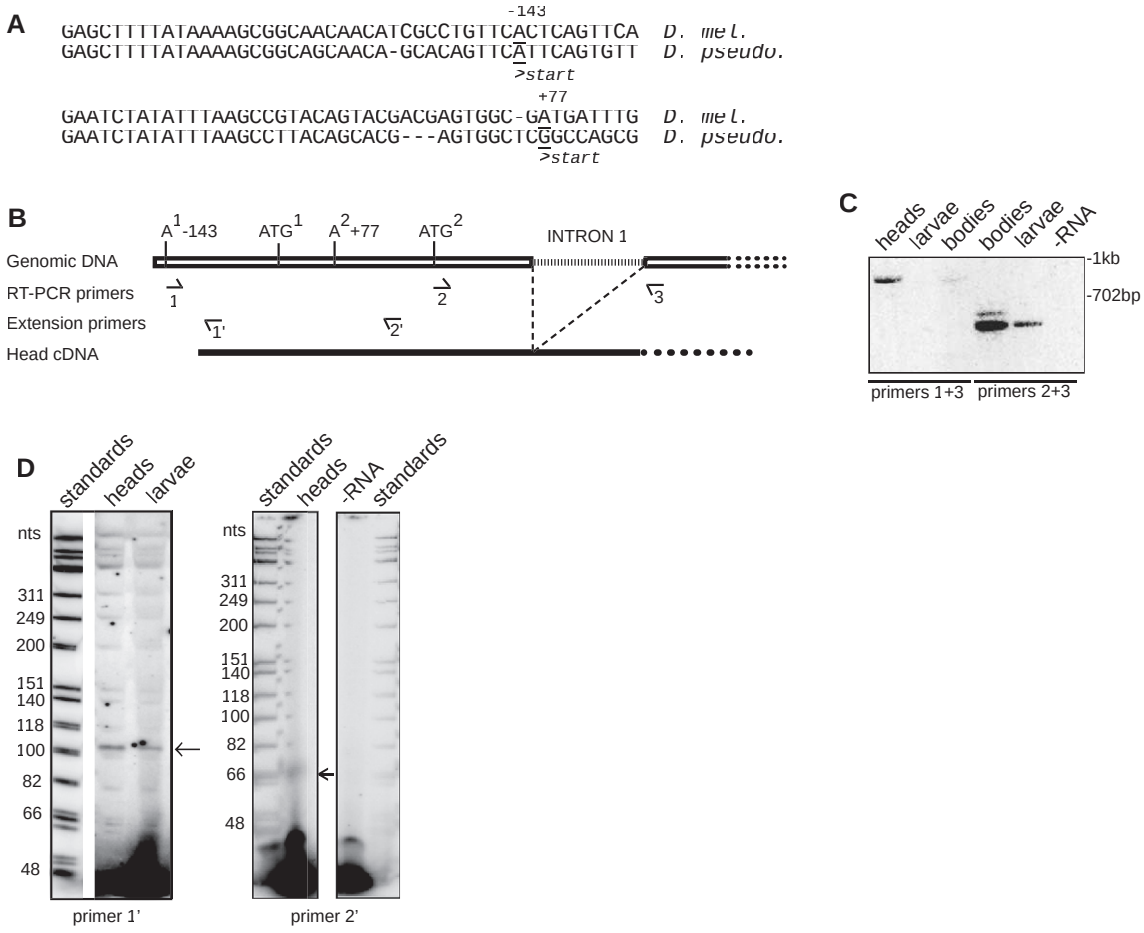


Fig. 2. Transcription from the *black/ Dgad2* locus. (A) Two putative transcription sites with equal probability values of 0.99, identified in the promoter/5′ UTR of the *black/Dgad2* gene in *D. melanogaster*, using the Berkeley Drosophila Genomes Project (BDGP) analytical tools program (Reese, 2001).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

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

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

3.2. The Dgad2 transcripts

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

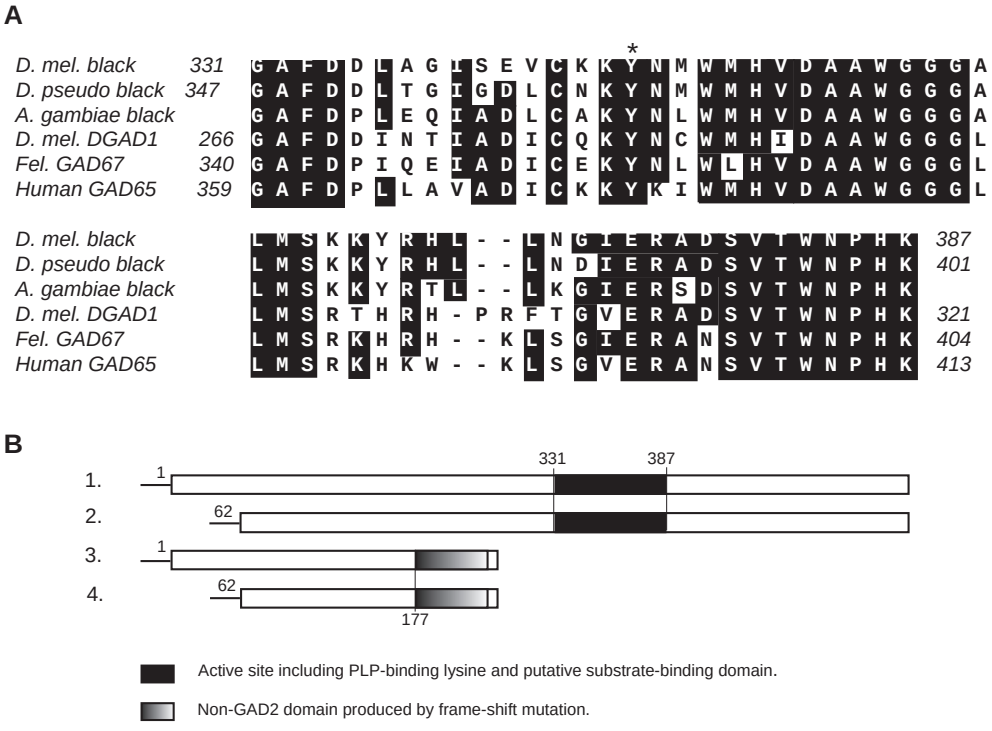


Fig. 3. Effect of the mutations in *black*^{1} on the encoded protein. (A) Alignment of the active site of *black/DGAD2* from *D. melanogaster, D. pseudoobscura* and *A. gambiae,* and of the related PLP-dependent decarboxylases, *D. melanogaster* DGAD1 (Jackson et al., 1990), *Feline* Gad67 (Kobayashi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

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

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

3.3. Molecular analysis of the Dgad2 gene

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

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

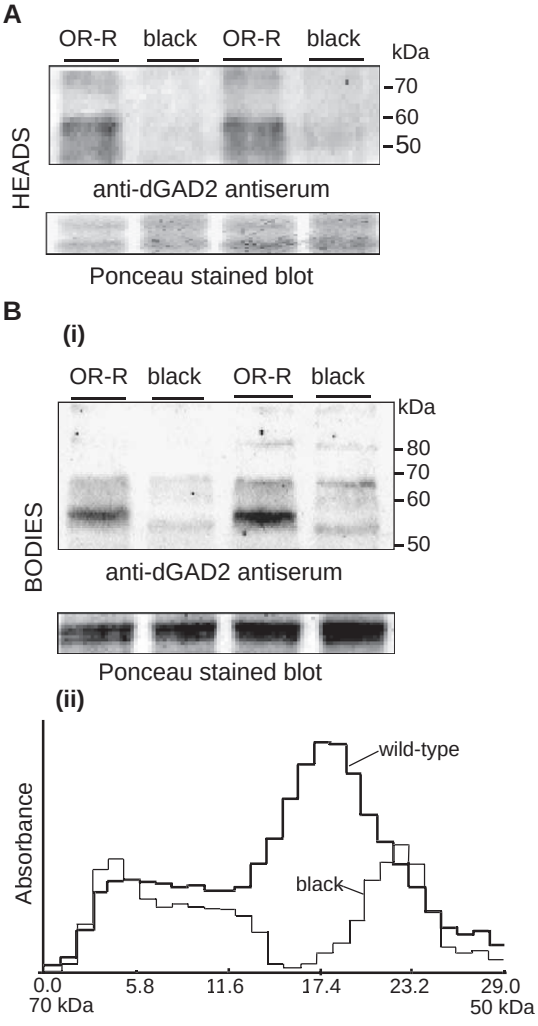


Fig. 4. The black/DGAD2 protein is absent from *black*^{1}. A: Western blots showing a protein at 58 kDa in the heads of adult wild-type flies that is not observed in the *black*^{1} mutant. The Ponceau stained panel is of the portion of the antibody probed filter, showing even loading. B: (i) Western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

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

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

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

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

3.5. Physiological assays and phenotypes

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

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

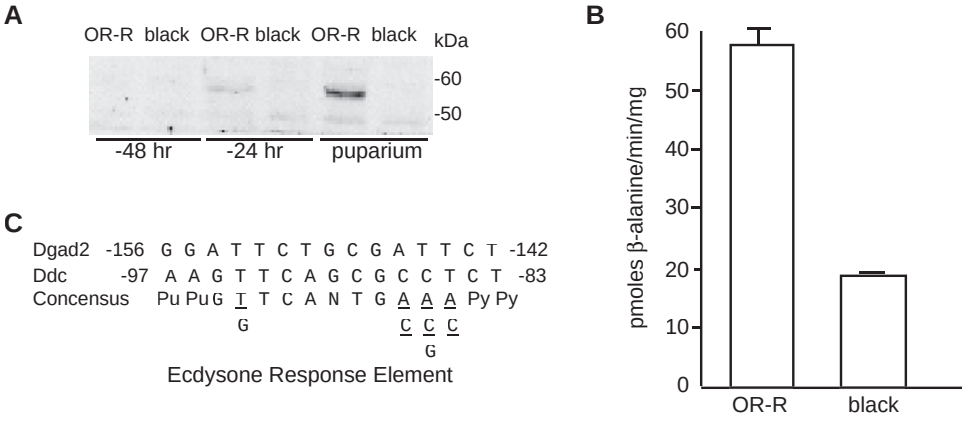


Fig. 5. DAGD2 at puparium formation. (A) Western blot, probed with affinity purified anti-black/Dgad2 antibodies, shows increasing levels of a 58 kDa protein in the 48 h up to pupation, in OR-R flies. (B) Aspartate decarboxylase assays using protein extracts from *black*^{1} and wild-type larvae at puparium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

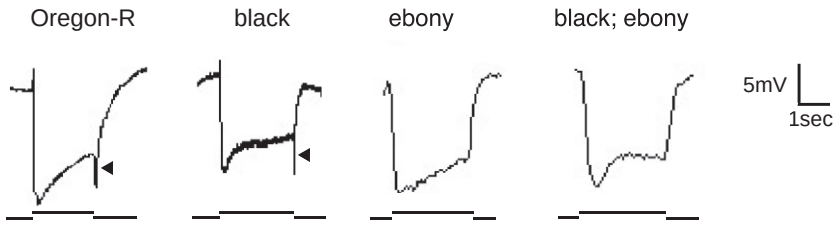


Fig. 6. Electroretinograms of the *black*^{1} mutant. Electroretinograms recorded from wild-type (Oregon-R), *black*^{1}, *ebony*, and *black*^{1}*; ebony* double mutant flies. The *ebony* flies show no transient responses at light-on or light-off indicating a failure of transmission from the photoreceptor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

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

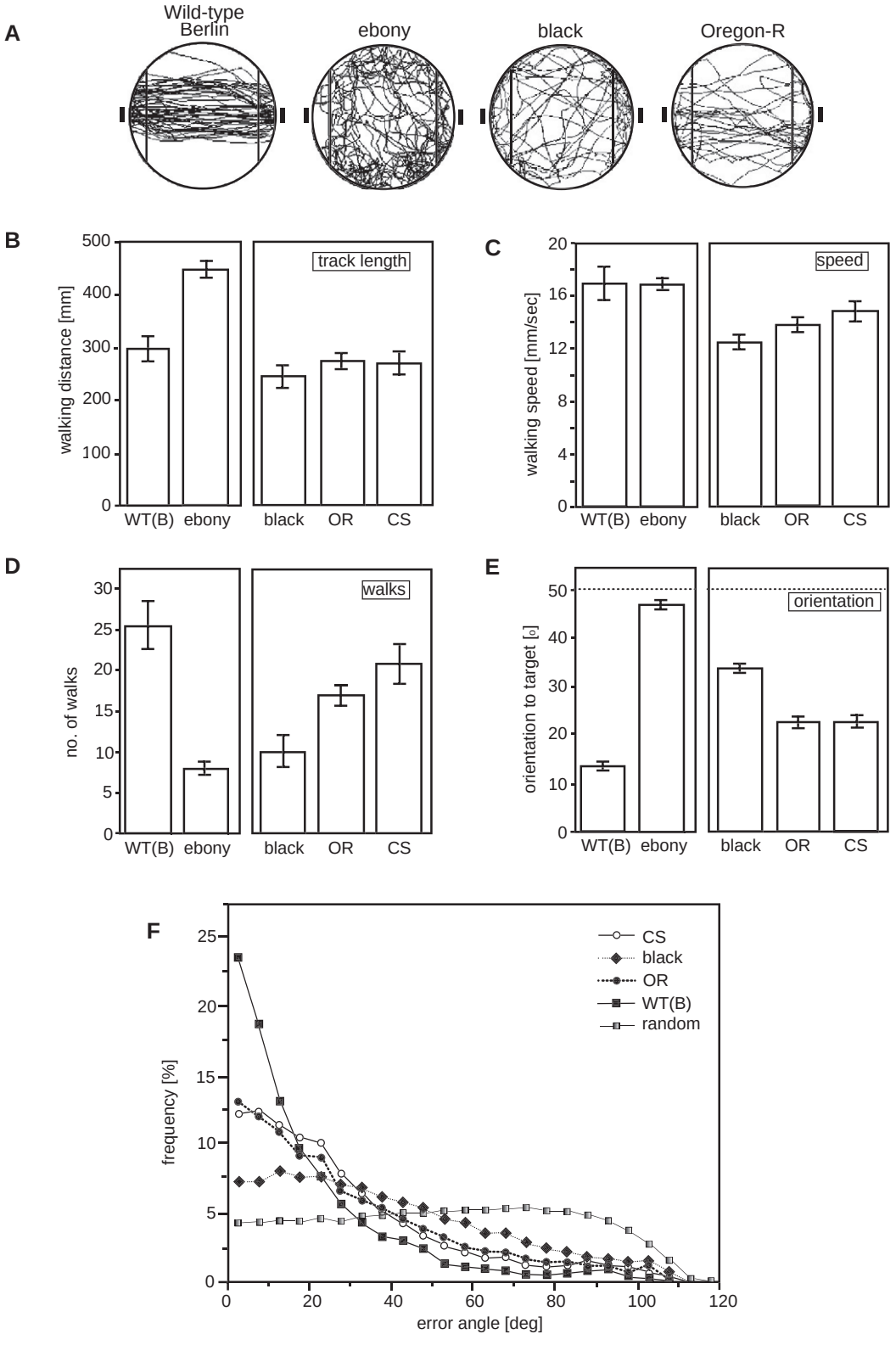
3.6. Aberrant orientation behaviour in *black*^{1} flies

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

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

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

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



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

4. Discussion

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

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

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

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

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

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

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

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

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

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