**Drosophila ABC transporter mutants white, brown and scarlet have altered contents and distribution of biogenic amines in the brain**

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**SUMMARY**

Monoamines such as dopamine, histamine and serotonin (5-HT) are widely distributed throughout the brain of the fruit fly *Drosophila melanogaster*, where many of their actions have been investigated. For example, histamine is released from photoreceptor synapses in the lamina neuropile of the visual system. Mutations of the genes *white*, an important eye pigmentation marker in fly genetics that encodes an ABC transporter, and its binding partner *brown*, cause neural phenotypes not readily reconciled solely with actions in eye pigmentation. We find that flies mutant for these genes, and another binding partner, *scarlet*, have about half the wild-type amount of histamine in the head, as well as reduced 5-HT and dopamine. These differences parallel reductions in immunoreactivity to the corresponding biogenic amines. They also correlate with the amine content of fractions after differential centrifugation of head homogenates. Thus, most of the amine is found in the vesicle-rich fraction of wild-type head homogenates, whereas it is found in the supernatant fractions from *white, brown* and *scarlet* flies. White co-expresses in lamina epithelial glia with Ebony, which conjugates histamine to β-alanine. Histamine is then released when the conjugate is hydrolyzed in photoreceptors, by Tan. Mutant *white* ameliorates the effects of *tan* on head histamine whereas it exacerbates the effects of *ebony*. Our results are consistent with the proposal that histamine uptake by the epithelial glia may be *white* dependent. Behavioral abnormalities in *white, brown* and *scarlet* mutants could arise because aminergic neurons in the *Drosophila* brain have reduced amine for release.

Key words: synaptic vesicle, photoreceptor, histamine, brain homogenate, cysteine string protein, HPLC.

**INTRODUCTION**

Screening pigments such as melanin in vertebrates (e.g. Oyster, 1999) or ommochromes and pteridines in insects (Phillips and Forrest, 1980) provide obvious means by which eyes screen their photoreceptor neurons from excess light or glare. As a result, corresponding pigmentation mutants, such as albino mammals and white-eyed *Drosophila melanogaster* have defective vision, especially at high light intensities. These defects are, however, only partly attributable to an inability to screen stray light. For example, albino mammals of many different species have neurological as well as retinal defects (Dräger and Balkema, 1987; Guillery, 1986; Jeffery, 1997). In *Drosophila*, although *white* mutants are positively phototactic their vision is not normal; they lack optomotor responses, for example (Kalms, 1943), and have an abnormal electroretinogram (ERG) (Wu and Wong, 1977). In other pigmentation mutants of *Drosophila*, neurological defects are not restricted to the visual system. Thus, mutants of the kynurenine pathway of tryptophan metabolism, a precursor in the biosynthesis of brown ommochome pigment (Phillips and Forrest, 1980), exhibit neural defects. The mutants *cardinal* (with excess 3-hydroxykynurenine) and *cinnabar* (with excess kynurenine) exhibit deficits in learning and memory and altered volumetric changes in the mushroom bodies of the brain (Savvateeva et al., 2000). *vermilion* mutants (which lacks kynurenes) suffer a gradual decline in learning and memory, and an increase in mushroom body volume (Savvateeva et al., 1999). These mutants also have decreased immunoreactivity to the cysteine string protein (CSP) (Zinsmaier et al., 1990), which functions at late stages of Ca²⁺-regulated exocytosis of synaptic vesicles (Dawson-Scully et al., 2000), implicating the action of both genes at synapses. Collectively, these examples indicate the pleiotropic action of pigmentation genes in the nervous system.

Pigmentation mutations have readily identifiable phenotypes. *white* was in fact the first genetic mutant to be isolated in *Drosophila* (Morgan, 1910), and its obvious eye phenotype leads to its widespread use as a genetic marker. Extreme *white* alleles and *white* deficiencies remove both brown and red pigments (Hadorn and Mitchell, 1951). Yet mutants of *white*, which encodes an ABC transporter, and its binding partner *brown* (Mount, 1987), have behavioral and other phenotypes not readily reconciled with an action in the eye. For example, volatile general anesthetics reveal behavioral differences attributable to neuronal action (Campbell and Nash, 2001), and possibly related, *white* mutants have reduced performance in spatial learning (Diegelmann et al., 2006). Our preliminary work provides evidence that, in addition to their action in loading pigment granules in the eye, White and its binding partners may be involved in a hitherto unappreciated transport function for biogenic amines (Borycz et al., 2005a).

In order to reveal a neural phenotype for *white* and its binding partner genes, we examined the neurotransmitter phenotypes of corresponding mutants. We could examine this most readily at one site in the fly’s visual system, the first optic neuropile, or lamina, where the synaptic terminals of photoreceptors from the compound eye use histamine as a neurotransmitter (Hardie, 1987), are also large, and vesicle-laden, and contain the highest concentration of the brain’s histamine (Borycz et al., 2005b).
MATERIALS AND METHODS

Animals

Adult fruit flies, Drosophila melanogaster Meigen, were from stocks held at 25°C in a 12 h:12 h light:dark cycle on a standard cornmeal and molasses medium. The following genotypes were used: Oregon R wild-type and corresponding eye color mutants, w^118 (a null white allele); bw^1 (a strong brown allele); s^1 (scarlet); e^1 (ebony); t^1 (tan), and double mutants: w^118/e^1 and w^118/t^1.

Wild-type Sarcophaga bullata (Parker) and the white-eyed ivory mutant of Sarcophaga barbata (Thomson), wild-type and the white-eyed chalky mutant of Calliphora erythrocephala (Meigen), and wild-type and white-eyed Musca domestica (L.), were all held at 24°C in a 12 h:12 h light:dark cycle and reared from larvae grown on commercial granulated laboratory rat food.

High-performance liquid chromatography

For determinations of brain biogenic amines, flies were collected, frozen at –80°C, shaken to decapitate them, and were then processed either for histamine determinations using high-performance liquid chromatography (HPLC) with electrochemical detection, as previously reported (Borycz et al., 2000) or, in parallel samples, for determinations of both dopamine and 5-HT. We used samples from: 50 heads (Drosophila), five heads (Musca) or one head (Calliphora and Sarcophaga) and between six and 12 samples per reported mean. For dopamine and 5-HT we also used HPLC with electrochemical detection. The chromatographic system consisted of a BAS PM-92e pump, BAS LC-22C temperature controller and an Epsilon amperometric detector (BAS, West Lafayette, IN, USA). The mobile phase contained (in mmol l–1): 9.1 monochloroacetic acid, 0.23 1-octane-sulphonic acid, 0.08 EDTA plus 1.5% acetonitrile (vol/vol) and 0.75% tetrahydrofuran (vol/vol), pH 2.3. Buffer was filtered through a 0.2 μm filter (Millipore, Bedford, MA, USA), and detected on a radial flow glassy carbon working electrode at an oxidation potential of +650 mV.

For histamine determinations using HPLC, four millilitres (100 mm × 2.1 mm) column coupled to an Alltech Adsorbosphere CAT 80 A, 3 μm guard column (7.5 mm × 4.6 mm: Alltech, Deerfield, IL, USA), and detected on a 10% Tris-HCl Ready Gel. Protein transfer was performed by electroblotting onto a pure nitrocellulose membrane (0.45 μm). After blocking with 5% bovine serum albumin (BSA), the nitrocellulose membrane was incubated for 1 h at 23°C with monoclonal anti-CSP antibody, diluted 1:2000 in Tris-buffered saline, 1% Tween 20. As a secondary antibody, peroxidase-conjugated goat anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA, USA; cat. no. 110-035-003) was used in the same buffered saline. The antibody detected a protein band at 34 kDa, which was identified as CSP.

Immunohistochemistry

Heads were fixed and embedded in OCT freezing solution (Sakura Finetechnical, Tokyo, Japan), frozen in liquid nitrogen and sections were cut in a frontal plane at 10 μm thickness on a cryostat (Reichert-Jung 2800, Frigocut: Leica Biosystems GmbH, Nussloch, Germany). The sections were processed for double immunolabeling with primary antibodies against the three neurotransmitters, according to previously published methods for histamine (Borycz et al., 2002), dopamine (Nässel et al., 1988) and 5-HT (Nässel et al., 1985), all used with monoclonal antibody 49-1 against synaptic vesicle protein, CSP, at a dilution of 1:100. The primary antibodies used, and their characterization, are listed in Table I. For White immunolabeling, brains were fixed in 4% formaldehyde, as paraformaldehyde (PFA) in 0.1 mol l–1 phosphate buffer (PB), and
immunolabeled at a dilution of 1:10 with a rabbit polyclonal antibody, raised against a synthetic peptide with the sequence: RYANEGLLINQWADVEPGEC, which represents a predicted extracellular loop of the White protein between putative transmembrane helices 5 and 6 of this protein (Ewart et al., 1994). Further details are given elsewhere (Mackenzie et al., 2000). For 5-HT immunolabeling, brains were fixed in 4% PFA in 0.1 mol l–1 PB, and immunolabeled with a mixture of rabbit polyclonal anti-serotonin (5-HT; Incstar, Stillwater, MN, USA) at a dilution of 1:500, and 49-1. For histamine immunolabeling, brains were fixed in 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (3h) and 4% PFA (2h), and then immunolabeled with a mixture of rabbit polyclonal antibody (PANI9C; ImmunoStar, Hudson, WI, USA) at a dilution of 1:500, and 49-1. For dopamine, brains were fixed in mixture containing 4% PFA, 1.6% glutaraldehyde, 1% picric acid and 10 mmol l–1 ascorbic acid in 0.1 mol l–1 PB. The sections were incubated in polyclonal antisera raised in rabbit anti-dopamine (ImmunoStar, Hudson, WI, USA) at a dilution of 1:500, and 49-1. All CSP and amine double-labelings were undertaken carefully in parallel using a single incubation for each of the four different genotypes, with at least one slide for each and six flies per slide. The following secondary antibodies were used: Cy-3-conjugated goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA, USA) at 1:400 and Alexa Fluor 488 goat anti-mouse (Molecular Probes, Eugene, OR, USA) at 1:100. Labeled sections were mounted in Vectashield beneath size 0 cover glasses and images collected with Zeiss LSM 410 or LSM 510 confocal microscopes, using Plan Neofluar glasses and images collected with Zeiss LSM 410 or LSM 510.

Table 1. Antibody documentation and characterization

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Host</th>
<th>Source</th>
<th>Cat. no.</th>
<th>Immunogen or peptide sequence</th>
<th>Test of specificity</th>
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<tbody>
<tr>
<td>To White</td>
<td>Rabbit</td>
<td>Dr Gary Ewart</td>
<td>–</td>
<td>RYANEGLLINQWADVEPGEC</td>
<td>No labeling in white&lt;sup&gt;111&lt;/sup&gt;B</td>
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<td></td>
<td>(Mackenzie et al., 2000), western blot</td>
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<tr>
<td>To histamine</td>
<td>Rabbit</td>
<td>ImmunoStar, Hudson, WI, USA</td>
<td>22939</td>
<td>Histamine coupled to KLH with carbodiimide</td>
<td>No labeling in hdc&lt;sup&gt;2910&lt;/sup&gt;</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td>(Melzig et al., 1996)</td>
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<tr>
<td>To serotonin</td>
<td>Rabbit</td>
<td>Incstar IR, Stillwater, MN, USA</td>
<td>20080</td>
<td>5-HT coupled to BSA with PFA</td>
<td>No labeling in ddc</td>
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<td></td>
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<td></td>
<td></td>
<td>(Vallés and White, 1986)</td>
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<tr>
<td>To dopamine</td>
<td>Rabbit</td>
<td>ImmunoStar, Hudson, WI, USA</td>
<td>22230</td>
<td>Dopamine coupled to BSA with glutaraldehyde</td>
<td>No labeling in ddc</td>
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<td>(Geffard et al., 1984)</td>
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<tr>
<td>To DCSP 1 (49-1)</td>
<td>Mouse</td>
<td>Dr Konrad Zinsmaier</td>
<td>–</td>
<td>Drosophila head homogenate</td>
<td>No labeling in mutant csp</td>
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<td>(Zinsmaier et al., 1990)</td>
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DCSP, *Drosophila* cysteine string protein; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; PFA, paraformaldehyde.

Throughout, values of biogenic amines are expressed as means ± s.d. of the mean values for 6–12 independent samples of head amine determinations, or for six independent samples of vesicle fractions, or for one or two independent samples for <sup>3</sup>H emissions, and for synaptic vesicle counts as means ± s.d. of the mean values for three flies. To compare organelle counts, as well as head contents, and pellet:supernatant contents of biogenic amines between wild-type and mutant flies, we used ANOVA followed by a Tukey’s HSD test, making use of software (Systat 5.2.1). To compare the amount of biogenic amines in pellet and supernatant fractions we used paired t-tests using the same software.

### Results

Our initial observations began when we used our methods (Borycz et al., 2000) to undertake routine determinations of head histamine in various *Drosophila* mutants that had been isolated in a genetic background containing the mutant white gene as a marker. These loss-of-function white-eyed mutants lack filled pigment granules in the eye but were thought otherwise to be normal. Our subsequent
findings clearly revealed that white is not normal, however, either for histamine or other biogenic amines.

**white, brown and scarlet mutants have reduced head contents of biogenic amines**

The histamine content of white heads was 1.06 ng, 51% less than that of the wild-type heads (Fig. 1A). This difference was significant ($P<0.0005$). The mean weight per head, from 50 that were weighed from wild-type flies, was 87.3±14.5 g (mean±s.d.); that for white was 83.0±13.8 g, a difference that was not significant ($t$-test). The differences in head histamine in white when normalized to head weight were, as a result, still significant ($P<0.0001$, $t$-test). Given that Brown and Scarlet proteins are ABC binding partners of White we therefore next examined brown and scarlet mutants and found that brown heads had a histamine content of 0.92 ng, and scarlet a content of 1.07 ng, both also significantly less than wild-type. Thus, in both mutants the histamine content was roughly half that of wild-type flies, differences that were likewise significant ($P<0.0002$; ANOVA followed by Tukey’s HSD test).

Given these differences for histamine, we next examined heads of the same mutants for dopamine and 5-HT, with similar results. In the case of dopamine, wild-type heads had a mean content of 678 pg, relative to which white mutants had 60% less, brown similarly 56% less and scarlet 40% less, differences that were statistically significant ($P<0.0005$) (Fig. 1B). For 5-HT, wild-type heads had 203 pg, relative to which white mutants had 52% less, whereas brown had 45% less, and scarlet 37% less, all significantly different from wild-type ($P<0.0005$; Fig. 1C). These differences in head amines in white when normalized to head weight were, like those for histamine, significant ($P<0.0001$, $t$-test).

The common feature of these findings was therefore that, relative to the wild-type heads, all three biogenic amines were reduced in white, brown and scarlet mutants. The significant reductions in all three mutants were between 30 and 60%. Thus, these changes were both clear and specific for each mutant and amine; however, for all these findings there was considerable variation in our determinations.

Since the total head content of neurotransmitter gave only an overall measure of mutant action, we next sought further details and a location.

**Mutants have reduced immunoreactivities to histamine and CSP**

Given these differences in overall head content, we proceeded to seek any differences in distribution of immunoreactivity to histamine in frontal cryostat sections of the heads of white, brown and scarlet, relative to wild-type flies. These are shown with respect to the immunolocalization of a synaptic vesicle protein, cysteine string protein (CSP) (Zinsmaier et al., 1990; Eberle et al., 1998), for histamine (Fig. 2). For histamine the strongest signal appeared in the visual system (Pollack and Hofbauer, 1991), which also showed a strong signal for 5-HT (Nässel, 1987). Dopamine showed very little labeling in the optic lobe, however, as previously reported (Nässel et al., 1988), for which reason we compared immunolabeling to this amine in the region of the central brain of frontal sections.

Consistent with the differences seen in histamine content for the entire head (Fig. 1), the signal strengths for histamine immunoreactivity in brains of all three mutants were reduced compared with wild-type fly brains (Fig. 2A,D,G,J). There were, however, some differences between the mutants. Thus white and brown flies showed drastically reduced histamine signal in the retina (Fig. 2D,G), whereas the histamine immunolabeling in scarlet mutants was not clearly different from that in the wild-type (Fig. 2A,J). All three mutants, especially white and brown, also had reduced immunoreactivity to the synaptic vesicle marker, CSP, with no CSP signal in the retinas of white and brown flies (Fig. 2E,H). The reduced signal in the retina proper probably resulted because the mutants lacked autofluorescence from red pigment in the eye. In fact, flies with a white marker are often used for convenience, to reduce this autofluorescence when immunolabeling in many antibody studies of the *Drosophila* brain. The reduced CSP immunosignal in the lamina is an interesting additional finding, because no a priori reason exists to link synaptic vesicles to the binding partners of an ABC transporter. In the wild-type lamina the histamine signal overlapped that for CSP, consistent with the vesicular sequestration of neurotransmitter. By contrast, in the laminas of all three mutants this pattern of colocalization was less complete.

For reasons that will become clear later, we also evaluated the distribution of histamine in the double mutant white; ebony (Fig. 2P), for which we also needed to examine single mutant ebony (Fig. 2M) as a comparison. The double mutant had visibly reduced
immunoreactivity to both histamine and CSP (Fig. 2P,Q,R) when compared with either ebony or white single-mutant controls. This suggests an additive function of the phenotype for both genes in the double mutant.

**Histamine content of the visual system**

Based on these findings, we next quantified the histamine content of the visual system and brain of the white mutant, in order to determine the sites of histamine loss. We found that the fresh whole-head content of histamine was 1.07 ng in the white mutant, 54% of the wild-type value reported by Borycz et al. (Borycz et al., 2005b). Of this, 0.85 ng survived after freeze-drying (47% of that in freeze-dried wild-type); of which the microdissected lamina contained 0.16 ng (64% that of the wild-type) the retina contained 0.43 ng (66% that of the wild-type), and the central brain 0.21 ng (63% that of the wild-type). Thus all three components had a similar reduction in histamine in the mutant but the lamina had a somewhat disproportionate loss. In principle, this loss could have been vesicular or cytoplasmic, because the total histamine determined from HPLC would not distinguish between these two components. Our next step was therefore to examine the synaptic vesicle population, to see if this too differed in the three mutants.

**white, brown and scarlet mutants also have fewer photoreceptor synaptic vesicles**

In general, the cytoplasmic concentration of neurotransmitters is low compared with the concentration in synaptic vesicles, for example with a ratio in the order of 1:100 for cholinergic synapses (Parsons et al., 1993) or perhaps an order of magnitude less than this for histamine in Drosophila photoreceptors (Borycz et al., 2005b). The reduced content of biogenic amines, and the altered distribution of these amines in the heads of white, brown and scarlet mutants suggested either that the synaptic vesicles themselves were fewer or that synaptic vesicles had reduced amounts of neurotransmitters, or both. To examine these alternatives, we therefore first needed to make counts of the synaptic vesicle populations in wild-type and mutant synaptic terminals. This was routinely possible only for the histaminergic terminals of the photoreceptor terminals R1–R6 in the lamina (Borycz et al., 2005b).

There were about 120 vesicle profiles per wild-type terminal profile in cross section, but the terminals of white and brown flies showed between 35% and 65% fewer, differences that were significant at \( P < 0.03 \) (\( t \)-test; Fig. 3E).

Thus the reduced number of synaptic vesicles roughly matched the lower head histamine content in white, brown and scarlet mutant R1–R6 terminals, and also corresponded to the reduced immunolabeling for CSP in the lamina (Fig. 2E,H,K) relative to that of the wild-type (Fig. 2B). To be sure that these differences were not attributable to differences in the packing density of vesicles, we also measured the cross-sectional areas of the R1–R6 profiles (Fig. 3A) to derive the profile packing density of vesicle profiles per square
mutant values (D,G,H).

which we attribute to our methods for sampling organelle profiles at relatively low frequencies. By chance, the variation appeared larger in wild-type than standard error in the wild-type mean.

although this difference was not significant because of the large depth of our samples, none was seen in the sampled (Meinertzhagen and Sorra, 2001). Perhaps as a result of in the lamina’s depth, so that their numbers alter with the depth of the synaptic vesicle population, the sites of histamine release, at tetrad synapses (Meinertzhagen and O’Neil, 1991), did not differ in number among the four genotypes, nor did their number per micrometer of membrane perimeter (Fig. 3C). This conservation has recently been reported for a wide range of other genotypes (Hiesinger et al., 2006). By comparison, the numbers of feedback synapses were far more variable in our samples. These genotypes (Hiesinger et al., 2006). By comparison, the numbers of feedback synapses were far more variable in our samples.

In contrast to the synaptic vesicle population, the sites of histamine release, at tetrad synapses (Meinertzhagen and O’Neil, 1991), did not differ in number among the four genotypes, nor did their number per micrometer of membrane perimeter (Fig. 3C). This conservation has recently been reported for a wide range of other genotypes (Hiesinger et al., 2006). By comparison, the numbers of feedback synapses were far more variable in our samples. These are mostly from lamina amacrine cells (Meinertzhagen and O’Neil, 1991; Meinertzhagen and Sorra, 2001) and are distributed unevenly in the lamina’s depth, so that their numbers alter with the depth sampled (Meinertzhagen and Sorra, 2001). Perhaps as a result of the depth of our samples, none was seen in the brown mutant, although this difference was not significant because of the large standard error in the wild-type mean.

Vesicle-enriched fractions from white, brown and scarlet brains contain altered biogenic amine contents

Next, we wished to address more directly the amine content of synaptic vesicles, to see whether the synaptic vesicle populations we had numerically characterized from the terminals of R1–R6 were typical of vesicles containing the other amines elsewhere in the fly’s brain, and whether their contents differed from wild-type vesicles. For this we fractionated fly head homogenates by centrifugation, and obtained a pellet fraction that contained cellular debris enriched in synaptic vesicles and other synaptic organelles. Our EM observations confirm that synaptosomes with synaptic vesicle profiles, some with capitate projection profiles typical of R1–R6 were present in the pellet fraction (Borycz et al., 2005a) (J.A.B., J.B., E. Pyza and I.A.M., manuscript in preparation). From these corresponding pellet and supernatant fractions we then determined their biogenic amine content to examine the partition of neurotransmitter between the vesicle-enriched pellet and supernatant fractions. Consistent results were obtained only after careful standardization of homogenization and fractionation procedures: all homogenizations were made in an ice-cold bath, using 10 strokes of the pestle and a homogenization buffer that was always freshly prepared.

white, brown and scarlet have reduced brain biogenic amines

(A,B) Sizes of R1–R6 profiles. Relative to wild-type controls, no differences were detected in profile sizes, either in their cross-sectional area (μm²; A) or membrane perimeter (μm; B). (C,D) The number of synapse profiles per micrometer of membrane perimeter, counted as either tetrad (C) or feedback (D). (E) Number of synaptic vesicle profiles per R1–R6 profile. These are significantly higher in Oregon-R wild-type than all mutant R1–R6 (P<0.03, t-test). (F–H) Numbers of capitate projections (CP) in the same samples as C–E, seen as three profiles: single penetrating (F), shallow (G) and multiple headed (H). (For definition of types, see Materials and methods.) No significant differences were seen except in the number of multiple-headed penetrating invaginations, which was greater in the wild-type control than in either white or scarlet heads (P<0.05, t-test, in H). Some counts showed wide variation, which we attribute to our methods for sampling organelle profiles at relatively low frequencies. By chance, the variation appeared larger in wild-type than mutant values (D,G,H).

micrometer. By contrast to the absolute numbers of synaptic vesicles, the profile density was in all cases about 40–50 per μm² and did not differ significantly between wild-type and mutant terminals. Thus the reduced number of synaptic vesicles in R1–R6 of white, brown and scarlet mutants must have been offset by an altered terminal cross-sectional area, even though these differences themselves were not significant.

Fig. 3. white, brown and scarlet mutants have organelle counts that mostly do not differ from wild-type controls. All values are mean ± s.d. (N=4 flies). (A,B) Sizes of R1–R6 profiles. Relative to wild-type controls, no differences were detected in profile sizes, either in their cross-sectional area (μm²; A) or membrane perimeter (μm; B). (C,D) The number of synapse profiles per micrometer of membrane perimeter, counted as either tetrad (C) or feedback (D). (E) Number of synaptic vesicle profiles per R1–R6 profile. These are significantly higher in Oregon-R wild-type than all mutant R1–R6 (P<0.03, t-test). (F–H) Numbers of capitate projections (CP) in the same samples as C–E, seen as three profiles: single penetrating (F), shallow (G) and multiple headed (H). (For definition of types, see Materials and methods.) No significant differences were seen except in the number of multiple-headed penetrating invaginations, which was greater in the wild-type control than in either white or scarlet heads (P<0.05, t-test, in H). Some counts showed wide variation, which we attribute to our methods for sampling organelle profiles at relatively low frequencies. By chance, the variation appeared larger in wild-type than mutant values (D,G,H).
prepared (Fig. 4). To confirm that the pellet fraction is enriched in synaptic vesicles, we used an antibody against CSP, a protein that co-purifies with synaptic vesicles (van de Goor et al., 1995) and is associated with synaptic vesicle membranes (van de Goor and Kelly, 1996). In western blots of wild-type head homogenate fractions an antibody against CSP recognized a clear band at ~34 kDa for the pellet fraction but failed to recognize such a band in the supernatant (Fig. 5). The antibody, 49-1 against Drosophila cysteine string protein (DCSP 1), detects four CSP protein isoforms of approximately 32, 33, 34 and 36 kDa (Zinsmaier et al., 1994), but separation between these is difficult to resolve using mini-gels (K. E. Zinsmaier, personal communication), and was not influenced by the previous freezing of the head required to make homogenates, because the same bands were seen using homogenates of fresh heads.

In the wild-type homogenates most of the neurotransmitter was found to be distributed in the vesicle-enriched pellet fraction (Fig. 4A). The pellet fraction contained on average 72% of total histamine, 92% of total dopamine, and 67% of total 5-HT, establishing for the wild-type control pellet:supernatant ratios of 2.57:1, 13.1:1 and 2.03:1, respectively. Relative to these, the corresponding values for all three mutants were reversed, at 0.64:1, 0.39:1 and 0.28:1 for white; 0.51:1, 0.39:1 and 0.72:1 for brown; and 0.57:1, 0.43:1 and 0.18:1 for scarlet (Fig. 4B).

From these findings, we may generalize the effects of all three mutant gene products: to cause a redistribution of the three amines from an organelle-bound compartment to a supernatant compartment. This redistribution suggests that in the absence of white gene function or its binding partners, there is a failure to pump the corresponding amine into a compartment contained within the pellet fraction of brain homogenates. The partition between pellet and supernatant was obviously specific for each particular mutant and amine. The lowest ratio was seen for 5-HT in scarlet, 0.18:1; and the highest ratio for dopamine in wild-type, 13.1:1 (Fig. 4B).

Moreover, often a considerable amount of neurotransmitter remained in the supernatant, presumably from cytoplasmic sources and because synaptic vesicles rupture during homogenization and fractionation. Conversely, some neurotransmitter remained in the pellet in mutant flies, possibly in synaptic vesicles but also in the contaminating cytoplasm or the contents of other organelles. The exact contributions from these two sources presumably depended on the action of the particular gene and the number and distribution of neurons containing the particular amine, as well as their structural integrity after homogenization. In the case of 5-HT in white mutants, at least, there must have been very little intravesicular amine. The supernatant fraction contained least dopamine and most histamine in wild-type fractions, suggesting that more histamine was liberated from ruptured vesicles in histaminergic synapses, than serotonergic and especially dopaminergic ones.

**white, brown and scarlet mutants have reduced numbers of capitate projections**

Capitate projections were previously shown to be sites of endocytosis of vesicle membrane, and these glial invaginating organelles have also been postulated to act as integrated sites not only for membrane
white, brown and scarlet have reduced brain biogenic amines

In view of the histamine phenotype in white, brown and scarlet flies, we therefore sought to examine whether these mutants also exhibited altered populations of capitate projections. The latter are dynamic organelles that have previously been shown to exist in one of two forms, shallow or penetrating. Shallow profiles are believed to be penetrating capitate projections during the process of either invagination into, or retraction from, the interior of the photoreceptor terminal (Pyza and Meinertzhagen, 1997). Penetrating capitate projections have either single heads or, far less frequently, multiple heads, with some overlap between the two profile types resulting from the plane of section. In comparing the photoreceptor terminals of white, brown, scarlet and wild-type flies, we found no significant difference in the normalized number of single-headed capitate projection profiles (Fig.3F), but differences in the number of multiple-headed profiles (Fig.3H), which were more numerous in wild-type. These differences were significant at \( P < 0.05 \) for white and scarlet, but not brown mutants. These differences, fewer synaptic vesicles and capitate projection profiles in the mutants, are consistent with, but offer no clear proof of, altered endocytotic retrieval and possibly also histamine recycling at R1–R6 photoreceptor terminals in the lamina.

**White protein is expressed in the lamina epithelial glia**

In order to gain a better understanding of the functioning of white, we next examined the localization of the White protein. Using a polyclonal antibody raised against a predicted intravesicular loop of the White protein, expression of white has previously been reported in granules of the pigment cells in the ommatidia (Mackenzie et al., 2000). Our labeling indeed confirms this pattern (Fig.6A). After immunocytochemical labeling with the same antibody we also found a distinct pattern of labeling in the wild-type that was strong in the retina but there was additional labeling, particularly in the underlying lamina, where the signal was even stronger (Fig.6A). In the eye, as previously reported (Mackenzie et al., 2000), the signal was concentrated in pigment cells (Fig.6A), but there was weaker signal as well in the photoreceptors themselves, which contain additional pigment granules. The pattern in the lamina, which has not previously been reported, was punctate and readily attributable to the epithelial glia that ensheathe the cartridges (Fig.6A, inset). The weak label in the photoreceptors was visible in the terminals of R1-R6 in the lamina, lying within the circle of epithelial glia, and also in the terminals of the other two photoreceptors, R7 and R8, which innervate the distal medulla (Fig.6A,B). Thus the pattern corresponds to the distribution of the pigment in the retina, as previously shown (Mackenzie et al., 2000).
White was almost entirely absent in the immunosignal in the medulla and central brain was extremely low and diffuse, with no clear cellular immunolabeled structures.

Relative to its distribution in the wild-type, immunoreactivity to White was almost entirely absent in the white null mutant (Fig. 6C). This lack confirms the specificity of the antibody labeling for the wild-type. A similar absence of signal was also seen in the brown mutant (Fig. 6D), whereas the scarlet mutant had a pattern that was relatively strong in the retina but greatly reduced in the lamina (Fig. 6E). These differences conform in a general way with those for the total head content of histamine, but also indicate that scarlet shows some distributional differences from white and brown. These may result either because the stl allele is hypomorphic or the Scarlet protein has a somewhat different function. Lack of mutant immunoreactivity in the lamina is consistent with the hypothesis that Brown is the binding partner of White in epithelial glia, and is necessary for its correct localization.

**White has a reciprocal effect on histamine in tan and ebony mutants**

In addition to white, epithelial glia also express another important regulator of histamine, the product of the ebony gene (Richardt et al., 2002), which is required to conjugate histamine to β-alanine (Borycz et al., 2002; Richardt et al., 2003). The β-alanyl conjugate, called carcinine (Borycz et al., 2002) is then hydrolyzed by the product of the tan gene, which is expressed in the photoreceptors (True et al., 2005; Wagner et al., 2007), the two genes forming a partnership that both expresses and acts reciprocally, so as to constitute a shuttle pathway that operates between photoreceptor and glial cell to recycle histamine (Stuart et al., 2007). Carcinine has recently been demonstrated to be taken up across the photoreceptor membrane by the product of inebriated (Gavin et al., 2007) but other transporters have not yet been identified, that either take up histamine at the photoreceptor terminal or extrude carcinine from the epithelial glia. The colocalization of White and Ebony in the epithelial glia and the high supernatant concentration of histamine in fractions from white, scarlet and brown mutants suggested to us that White may act somewhere in the pathway for histamine recycling via the epithelial glia. To test this possibility, we made double-mutant flies for white with either tan or ebony. We found that white significantly offset the effect of tan in reducing head histamine. Relative to the 2.0 ng of histamine in the wild-type head, tan mutant heads had 0.20 ng, whereas white, tan double mutants had 0.69 ng. Thus, head histamine was only reduced in the double mutant to 34% the wild-type value (Fig. 7), significantly less of a reduction than in tan (P<0.0005), which has less than 10% (Borycz et al., 2002). By contrast, white significantly exacerbated the effect of ebony (Figs 2 and 7). As a result, white; ebony mutants had 0.38 ng of histamine in the head, only 19% of wild-type value, significantly less than in ebony alone (P<0.0005), which had 0.97 ng, about half the wild-type value (Borycz et al., 2002).

These findings indicate that white interacts with both tan and ebony, compatible with it acting in the pathway for histamine recycling. To examine that possibility more closely, we gave the double-mutant flies, white, tan and white; ebony, sugar water to drink that was laced with [3H]histamine. Both the single mutant white and double mutant, white, tan flies took up and converted far less histamine to carcinine than did tan flies (Fig. 8A), as if white mutants for some reason had considerably reduced access to the exogenous [3H]histamine. The difference between the [3H]carcinine peaks was significant (P<0.002). With ebony, greatly reduced [3H]histamine uptake was observed, compared with that of the wild-type, but when this was combined with white in the double mutant white; ebony the amount of [3H]histamine taken up was considerably greater (Fig. 8B), a difference that was significant (P<0.01). The greater uptake of [3H]histamine in the double mutant obviously did not contribute to histamine in the visual system, which was greatly diminished (Fig. 2P) compared with that of either ebony (Fig. 2M) or white (Fig. 2D) single mutants. Thus if white acts to reduce histamine uptake, and if ebony when also mutant causes a failure to store histamine as carcinine, the uptake of [3H]histamine we found in the double-mutant white; ebony should occur outside the visual system. Although other interpretations are also possible, the results suggest that white may be involved in taking up histamine into the glia. Its role there is still not clear and is also not absolute. Thus, after 3 days in constant light, head histamine in white mutants is decreased by almost 40% but does not alter in the wild-type (J.B., unpublished data), suggesting that histamine recovery is reduced in white mutants but not completely blocked.

**White-eye mutants in other fly species also have reduced histamine in the head**

Finally, given the uniformity of white’s action in Drosophila, we sought to identify whether mutants with white eyes in larger fly species also have reduced amounts of biogenic amines in the head. Such spontaneous white-eyed mutants have been isolated in a number of species, and we examined samples from the housefly Musca domestica, the blowfly Calliphora erythrocephala and two species of the flesh fly Sarcophaga, S. bullata (wild-type) with S. barbata (ivory, white-eyed mutant) which constitute a series of increasing body sizes, to compare with data from the smaller Drosophila (Fig. 9). Although the genetic basis of white-eye mutants is known only in Drosophila, the mutants in the other species had similar defects in the amount of histamine in the head, which relative to the red-eyed wild-type were reduced significantly in all species (P<10^-6 in all cases, t-test). The reduction was more severe in proportion to body size, so that in the largest species, Sarcophaga, white-eyed flies had less than 30% of the histamine of their wild-type counterparts, possibly because their eyes are disproportionately large. The histamine phenotype in these mutants suggests that the

![Fig. 7. Histamine in the head is altered reciprocally by white, in double mutants with ebony and tan. In all single mutants, the histamine content is reduced, to 51% (white), 48% (ebony) and 10% (tan) of the wild-type value. Relative to these, double-mutant white; tan has significantly more histamine than the corresponding tan single mutant (P<0.0005), which has 34% of the wild-type value; whereas white; ebony has less head histamine than either single mutant alone, reduced to 19% of the wild-type value, a reduction that is significant (P<0.0005).](image-url)
that producing different eye pigmentation phenotypes. Here, we report partners (Ewart and Howells, 1998), Brown and Scarlet each white of the proteins with many cellular functions (Higgins, 1992). Specificity ABC transporters are paired heterodimer ATPase transporter locus in these other fly species may also be related to white in Drosophila.

DISCUSSION

ABC transporters are paired heterodimer ATPase transporter proteins with many cellular functions (Higgins, 1992). Specificity of the white gene product is largely determined by its binding partners (Ewart and Howells, 1998), Brown and Scarlet each producing different eye pigmentation phenotypes. Here, we report that white, brown and scarlet mutants not only lack normal pigment granule contents in their eyes, but also normal biogenic amines in their brains, apparently because their synaptic vesicle contents are altered. Thus all three mutant flies have different neurological phenotypes from the wild-type. Behavioral differences not attributable to eye coloration, but of neural origin, have been reported in these mutants (Campbell and Nash, 2001; Diegelmann et al., 2006) (M. Anaka, A. J. Haigh, C. D. MacDonald, E. Barkova, K. Simon, R. Rostom, I.A.M. and V.L., manuscript in preparation).

Possibly related to these, misexpression or mislocalization of mini-white, a truncated form of the white gene or wild-type white, generates altered sexual behavior in male flies (Zhang and Odenwald, 1995; Hing and Carlson, 1996) (M. Anaka, A. J. Haigh, C. D. MacDonald, E. Barkova, K. Simon, R. Rostom, I.A.M. and V.L., manuscript in preparation). Although it is not clear what, if any, behavioral features these examples might share, it is possible that all could be regulated by biogenic amines acting as neuromodulators, the release of which is reduced in white.

Dopamine and serotonin content in the Drosophila head

The amine levels in wild-type fly heads obviously vary, and this may also be true within mutant lines. Thus, our results for dopamine in the heads of white mutants are 33% higher, and for 5-HT 365% higher than data recently reported by Hardie and Hirsh (Hardie and Hirsh, 2006). These authors showed chromatograms of the separation of dopamine and 5-HT from wild-type flies, but not the measured values for each. We were therefore unable to compare our wild-type data with theirs. Recently, Sang et al. (Sang et al., 2007) reported approximately 300 pg/head for dopamine in a Ddce-GAL4 Drosophila line, which apparently had a w^{1118} mutant background (Li et al., 2000), a determination very similar to our data on this white mutant. By contrast, in another study (Dierick and Greenspan, 2007), basal levels of 5-HT in the head of Canton S average between 60–80 pg/head, 2.5 to 3.3 times less than our data. These differences could result from genotypic differences in the wild-type, but are more likely the outcome of dietary differences. Thus Drosophila fed with 50 mmol l–1 5-hydroxytryptophan, the immediate precursor of 5-HT, showed a 15- to 20-fold increase in 5-HT in the head (Dierick and Greenspan, 2007). Close standardization of the medium is thus required when analyzing 5-HT in the head to enable comparisons between different studies. Additional variables include sex and age. Thus, Neckameyer et al. (Neckameyer et al., 2000) report more dopamine in males than females, and in younger flies than older. These values refer to whole-body determinations of dopamine, however, not to heads, and although our samples were from 10 flies, our determinations are
reported for a minimum of eight samples taken from flies about 7-days old, so that overall we presume that they reflect both sexes and a spectrum of ages.

The contents of synaptic vesicles in mutant fly neurons

In brain homogenates we find that the partition between pellet and supernatant varies both for the particular amine and individual mutant. Intact neurons concentrate neurotransmitter in synaptic vesicles, by a factor of 100 at cholinergic synapses (Parsons et al., 1993) or lower, perhaps 8:1 (Borycz et al., 2005b) in Drosophila photoreceptors, which contain most of the photoreceptor histamine (Borycz et al., 2000). In brain homogenates, however, the equivalent pellet:supernatant ratio for histamine is only about 2.57:1, suggesting that neurotransmitter is lost from synaptic vesicles into the supernatant. This loss could result directly from vesicle damage during homogenization. An alternative, and in our view more likely, explanation is based on the rate of vesicle recycling, calculated for histamine release at R1–R6 (Borycz et al., 2005b; Stuart et al., 2007), which suggests that vesicle shedding may still have occurred in homogenates, so as to deplete histamine-containing organelles in the pellet. This rate in vivo is very rapid, sufficient to deplete the terminal by a calculated 11% of its histamine per second, if compensatory histamine recycling were not to occur (Stuart et al., 2007), and thus to deplete photoreceptor synaptosomes more severely than the synaptosomes of other neurons in the pellet. Release by vesicle shedding within the homogenate would shift histamine from pellet to supernatant, and plausibly follow structural disruption of epithelial glia, sites of ebonymph action (Stuart et al., 2007). Supporting this conclusion, the pellet:supernatant ratio is 13.1:1 for dopamine, indicating that retention of vesicular neurotransmitter in the pellet is high and that homogenization per se is non-destructive.

For the mutants, pellet:supernatant ratios are reversed, the wild-type:white mutant ratios for dopamine differing 34-fold, and for 5-HT, 7-fold. These differences suggest that most intravesicular amine found in the wild-type must be absent in the pellet fraction from the mutant. Other amines present in the pellet fractions from other mutants are sufficient to suggest either that only some synaptic vesicles are wholly depleted or that all are only partially depleted.

Each mutation acts specifically on the amine profiles of the brain. Compatible with its suggested role as one half of an ABC-type transporter (Ames, 1986; Mount, 1987), white has the most comprehensive overall action. Differences in the amine phenotype of each mutant are related to those for eye pigment granules. Thus white and brown flies fail to transport guanine (Sullivan et al., 1979), whereas white and scarlet have reduced uptake of tryptophan and kynurenine (Sullivan and Sullivan, 1975). Transport of both substrates is impaired in white mutants, but there is a broad spectrum of transport substrates, which our data now suggest may also include biogenic amines. For tryptophan, a precursor of 5-HT, we therefore anticipated reduced 5-HT in white and scarlet mutants, but in fact found no significant difference from brown mutants. We found instead a difference in head dopamine, between scarlet flies on the one hand, and white and brown flies, on the other. Each mutant has a neurotransmitter phenotype that we propose reflects the gene’s involvement in amine transport, and the physiology of the corresponding aminergic neurons.

Synaptic vesicles, pigment granules and the possible role of glia

A candidate point of convergence between the amine and pigment phenotypes of white and its binding partners could lie in their respective storage organelles, synaptic vesicles and pigment granules. Pigment granules (Summers et al., 1982) are ultimately vesicular elaborations of the Golgi apparatus (Shoup, 1966), and synaptic vesicles also arise from the trans-Golgi network (Regnier-Vigouroux and Huttner, 1993). Immunoreactivity to White and Scarlet localizes to the granule membranes (Mackenzie et al., 2000), and white-dsred tag colocalizes with the endosomal marker Garnet (M. Anaka, A. J. Haigh, C. D. MacDonald, E. Barkova, K. Simon, R. Rostom, I.A.M. and V.L., manuscript in preparation). Synaptic vesicles, which are serviced by AP-3 vesicles (Fáñdez et al., 1998) that transport White (Lloyd et al., 2002), might therefore be expected to express White. In the lamina, however, White localizes most strongly to epithelial glia, rather than synaptic vesicles.

The same epithelial glia that strongly express both white and ebonymph (Richard et al., 2002), also invaginate R1–R6 terminals at capitate projections, postulated sites for histamine recycling (Fabian-Fine et al., 2003) that have more multiple heads in mutant white terminals. Brown is a binding partner of White in the eye (Dreesen et al., 1988), and both brown and white mutants lack White expression in the lamina, as if the two may also be binding partners there. The lack in brown mutants suggests that White protein must first bind to Brown to localize correctly in the lamina. A similar interaction may be necessary to transport or stabilize the Scarlet–White dimer (Mackenzie et al., 2000). The functional outcome of white in the lamina is unclear, because the mutant differs from wild-type only in being more light-sensitive (Hengstenberg and Götz, 1967; Pak et al., 1969), reflecting the loss of pigment granules, but possibly also having impaired synaptic transmission.

Our data identify the interaction between White and Brown best for histamine in the lamina, but white must also function for the other amines, which show similar redistribution between pellet and supernatant fractions, consistent with a shift from organelle-bound storage. It is not clear why our data fail to reveal clear levels of White protein expression elsewhere in the brain. In situ hybridization likewise reveals white in the eye but not the brain, indicating that possible transcription in the brain must be at least an order of magnitude less (Fjose et al., 1984). However, RT-PCR does reveal reduced but clear expression of white in sine

![Image of histamine levels in fly species](image-url)
histamine as carcinine, which they cannot synthesize (Borycz et al., 2002), and thus have no way to retain ingested tritium, thus having less [3H]histamine than wild-type. According to the model for white, double-mutant white; ebony flies would be unable to take up histamine at the epithelial glia, and therefore could not store it at this site. We therefore propose that the increased [3H]histamine in white; ebony mutants reflects an uptake outside the visual system. We must acknowledge that the strength of our interpretation is circumscribed by such alternative expression sites for ebony and tan, by the histaminergic roles of additional lamina glia, and by the possibility that white might also have additional transport functions in epithelial glia. With these qualifications in mind we nevertheless predict a model in which white acts at the epithelial glia to take up histamine from the synaptic cleft of the photoreceptor (Fig. 10).

Significance of the white phenotype for fly genetics and behavior

Given their obvious pigmentation phenotypes, mutants of white and white transgenes have been widely used as genetic markers. One significance of our findings, therefore, is that many effects attributed to a mutant gene or transgene isolated in a white background may not simply be those of the unknown gene but also of white itself. This is particularly true for many new genes isolated in whole-eye mosaic flies produced by mitotic recombination (Stowers and Schwarz, 1999; Newsome et al., 2000). Our findings indicate that, as assayed in the synaptic terminals of photoreceptors, white and its binding partner mutants lack normal synaptic vesicle populations and vesicle contents. Although we have not localized similar changes in the other biogenic amines to neurons, our data reveal parallel deficits in these too. As a result, neurons may have reduced amine for release as either a neurotransmitter or neuromodulator, especially for sustained or high-output levels of transmission, leading to behavioral consequences. The exact behavior will reflect a balance between synthesis, transport and prior release rates of the particular amine. Thus, despite basic similarities, the behavioral phenotypes may vary both in the different mutants and, to some extent, under different physiological conditions.

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