The Journal of Experimental Biology 211, 3454-3466
Published by The Company of Biologists 2008
doi:10.1242/jeb.021162

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

J. Borycz[1], J. A. Borycz[1], A. Kubów[1], V. Lloyd[2,*] and I. A. Meinertzhagen[1,2,†]

[1]Department of Psychology and [2]Department of Biology, Life Sciences Centre, Dalhousie University, Halifax, NS, Canada B3H 4J1

*Present address: Department of Biology, Mount Allison University, 63B York Street, Sackville, NB, Canada E4L 1G7

†Author for correspondence (e-mail: iam@dal.ca)

Accepted 26 August 2008

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 (Kalmus, 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 ommochrome 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 kynurenines) 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 Ca^{2+}-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^{1118} (a null *white* allele); bw^{1} (a strong *brown* allele); st^{1} (*scarlet*); e^{1} (*ebony*); t^{1} (*tan*), and double mutants: w^{1118}; e^{1} and w^{1118}, 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), degassed and pumped through the system at a flow rate of 0.2 ml min^{-1}. Dopamine and 5-HT were separated on an Alltech Adsorbosphere CAT 80 A, 3 μm (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 radial flow glassy carbon working electrode at an oxidation potential of +650 mV *vs* Ag/AgCl. The analytical column was maintained at 32°C. For dopamine and 5-HT determinations the heads from 10 *Drosophila* about 1-week old were homogenized in ice-cold 0.1 mol l^{-1} perchloric acid, filtered through a 0.2 μm filter and injected into the HPLC system. To evaluate the recovery of amines, 300 pg of 3,4-dihydroxybenzylamine (RBI, Natick, MA, USA) was added to each sample. The amount injected was equivalent to the contents of one *Drosophila* head. The amounts of dopamine and 5-HT were calculated from the height of the peaks compared with standards. Insofar as dopamine is also a substrate for the process of melanization of insect cuticle (Wittkopp et al., 2002) we also dissected 50 Oregon R wild-type *Drosophila* heads that finally gave us five HPLC samples of brains, which lacked all cuticle and hypoderm, and five samples of the corresponding cuticle shells. Dissections were made in a droplet of 0.9% NaCl, in a Petri dish that was put in an ice-cold bath. The brains and remaining cuticles were processed for HPLC as described above. After dissections we observed 71% of the total whole head dopamine in the brain (wild-type mean content of 678 pg/head) and 15% in the cuticle. We assume that the remaining 14% of the dopamine was lost during dissection, either on dissecting instruments or from enzymatic degradation. These results indicate that most dopamine that we measured in our experiments from whole-head extracts did in fact originate from the brain.

Microdissection of freeze-dried heads

To determine the histamine contents of individual components of the visual system, fly heads were fixed on ice for 5 h in 4% 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide (E-7750; Sigma, St Louis, MO, USA) and immediately freeze-dried from acetone, as previously reported (Borycz et al., 2005b). Three different components were dissected from these brains using mounted tungsten needles, also as previously reported (Fujita et al., 1987; Borycz et al., 2005b). Histamine determinations were then made from the summed components of 20 heads dissected in this way, and the means of the mean values calculated for ten separate samples.

Head fractionation methods

To estimate the amount of vesicle-associated and non-vesicle-associated histamine, dopamine and 5-HT in the brain, and the ratio between these two compartments, we isolated synaptosome fractions using modifications of a published method (Tabb and Ueda, 1991), as previously reported (Borycz et al., 2005a). Flies were collected, frozen at –80°C, shaken to decapitate them, and the heads then sieved from the bodies through a wire mesh with a pore size of 425 μm. For this, samples of fly heads prepared as for HPLC determinations were homogenized in an aqueous solution containing 0.32 mol l^{-1} sucrose, 0.5 mmol l^{-1} calcium acetate, 1 mmol l^{-1} magnesium acetate, 1 mmol l^{-1} $NaHCO_3$ and a protease cocktail tablet (Roche, Indianapolis, IN, USA; cat. no. 11 836 153 001), centrifuged at 24,000 ***g*** for 20 min at 4°C. The supernatant was removed and its amine content determined, and the pellet was lysed in 0.1 mol l^{-1} perchloric acid and its amine content then also determined. The average recovery of histamine by these methods, the sum of both the pellet and supernatant fractions, was approximately 73%. For dopamine and 5-HT the recoveries were 68% and 64%, respectively.

Western blots

For immunoblots, supernatant and pellet fractions were mixed with 50 μl of Laemmli buffer (pH 6.8; Sigma, cat. no. 161-0737). Proteins within these fractions were then separated in the Ready Gel System (Bio-Rad Laboratories, Hercules, CA, USA; cat. no. 161-1101) using 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. 115-035-003) 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 1. For White immunolabeling, brains were fixed in 4% formaldehyde, as paraformaldehyde (PFA) in 0.1 mol l^{-1} phosphate buffer (PB), and

Table 1. Antibody documentation and characterization

Antibody	Host	Source	Cat. no.	Immunogen or peptide sequence	Test of specificity
To White	Rabbit	Dr Gary Ewart	–	RYANEGLLINQWADVEPGEC	No labeling in *white*^{1118} (Mackenzie et al., 2000), western blot
To histamine	Rabbit	ImmunoStar, Hudson, WI, USA	22939	Histamine coupled to KLH with carbodiimide	No labeling in *hdc*^{JK910} (Melzig et al., 1996)
To serotonin	Rabbit	Incstar IR, Stillwater, MN, USA	20080	5-HT coupled to BSA with PFA	No labeling in *ddc* (Vallés and White, 1986)
To dopamine	Rabbit	ImmunoStar, Hudson, WI, USA	22230	Dopamine coupled to BSA with glutaraldehyde	No labeling in *ddc* (Geffard et al., 1984)
To DCSP 1 (49-1)	Mouse	Dr Konrad Zinsmaier	–	*Drosophila* head homogenate	No labeling in mutant *csp* (Bronk et al., 2005), western blot (Zinsmaier et al., 1990)

DCSP, *Drosophila* cysteine string protein; KLH, keyhole limpet hemocyanin; BSA, bovine serum albumin; PFA, paraformaldehyde.

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 (3 h) and 4% PFA (2 h), and then immunolabeled with a mixture of rabbit polyclonal antibody (PAN19C; 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 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 ×40/1.4 (LSM410), ×40/1.3, ×63/1.4 or ×100/1.4 (LSM510) oil immersion objectives. Images of preparations from wild-type and mutant heads, double-labeled for CSP and amine, were collected using the same confocal operating parameters for brightness and contrast.

Electron microscopy and synaptic organelle counts

Heads were fixed and prepared for electron microscopy (EM) as previously reported (Meinertzhagen, 1996). Ultrathin sections cut in all cases at 50-nm thickness were examined at 80 kV using an FEI Tecnai 12 electron microscope. We used such sections to count the profiles of three types of synaptic organelles. The first were 30-nm synaptic vesicles, which surrounded the second organelle, T-shaped presynaptic ribbons at release sites of the photoreceptor terminal (Meinertzhagen and O'Neil, 1991). The third organelle type were capitate projections, which are specialized sites where surrounding epithelial glial cells invaginate the photoreceptor terminal (Stark and Carlson, 1986). Using previously established criteria (Pyza and Meinertzhagen, 1998) we counted these as either shallow (with a head invaginating not more than half its diameter), single (penetrating, with a single head profile) or multiple-headed (penetrating, with more than one head) profiles. To normalize these profile counts with respect to the size of the photoreceptor terminal, we also measured perimeters and cross-sectional areas of the terminals' profile using software (NIH Image).

Uptake of tritiated histamine

The method was adapted from our previous report (Borycz et al., 2002). Flies were dehydrated for 3 h, after which they were given a droplet of 25% [^{3}H]histamine (37 MBq ml^{-1} and 858.4 GBq mmol l^{-1}; Perkin-Elmer, Boston, MA, USA) in 4% aqueous glucose. After 40 min, flies were frozen, and their heads collected and prepared for HPLC as above. Samples were separated by HPLC, and fractions of the mobile phase collected at 1 min intervals. Samples of the mobile phase, 1 ml mixed with 5 ml of scintillation cocktail (Ready Safe; Beckman Coulter) were counted for 5 min in a scintillation counter (Beckman Coulter LS 6500). The retention time for [^{3}H]histamine and its metabolite tritiated-β-alanylhistamine ([^{3}H]carcinine) in these fractions was confirmed exactly from the retention time for the histamine and carcinine peaks seen by electrochemical detection. In case the peak straddled two samples, the quantity of ^{3}H in histamine and carcinine was measured by summing the two adjacent ^{3}H fractions.

Statistical analysis

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 ^{3}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

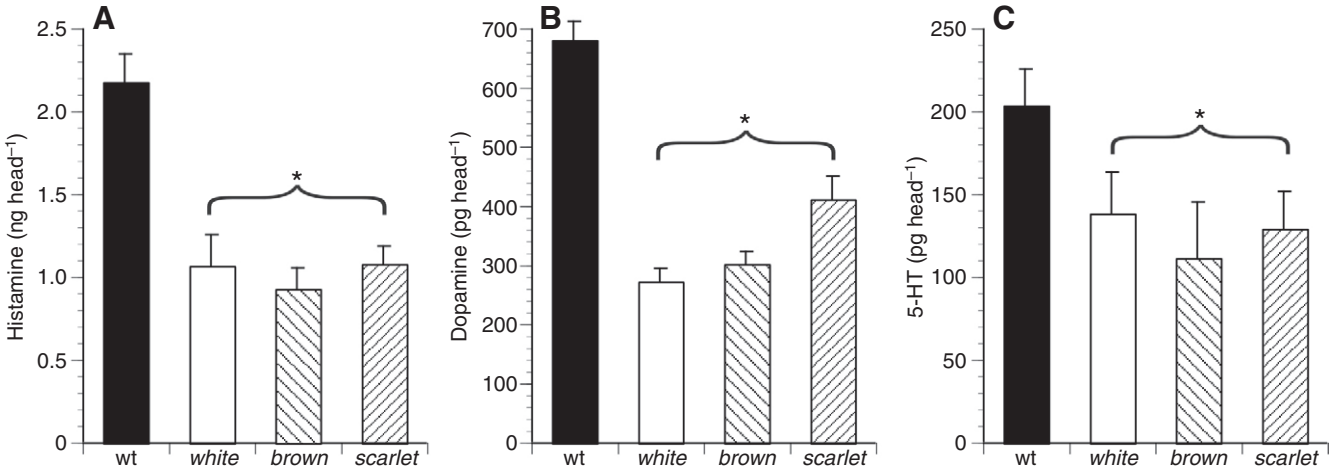

Fig. 1. Histamine, dopamine and 5-HT are reduced in the heads of *white*, *brown* and *scarlet* mutants relative to wild-type heads. (A) Histamine content, means of means from 10–13 samples for each genotype. (B) Dopamine content, means of means from 10 samples for each genotype. (C) 5-HT content, means of means from 10 samples for each genotype. All three mutants differ from Oregon-R wild-type for all three amines: **P*<0.0005 (*t*-test).

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 µg (mean±s.d.); that for *white* was 83.0±13.8, 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 32% 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

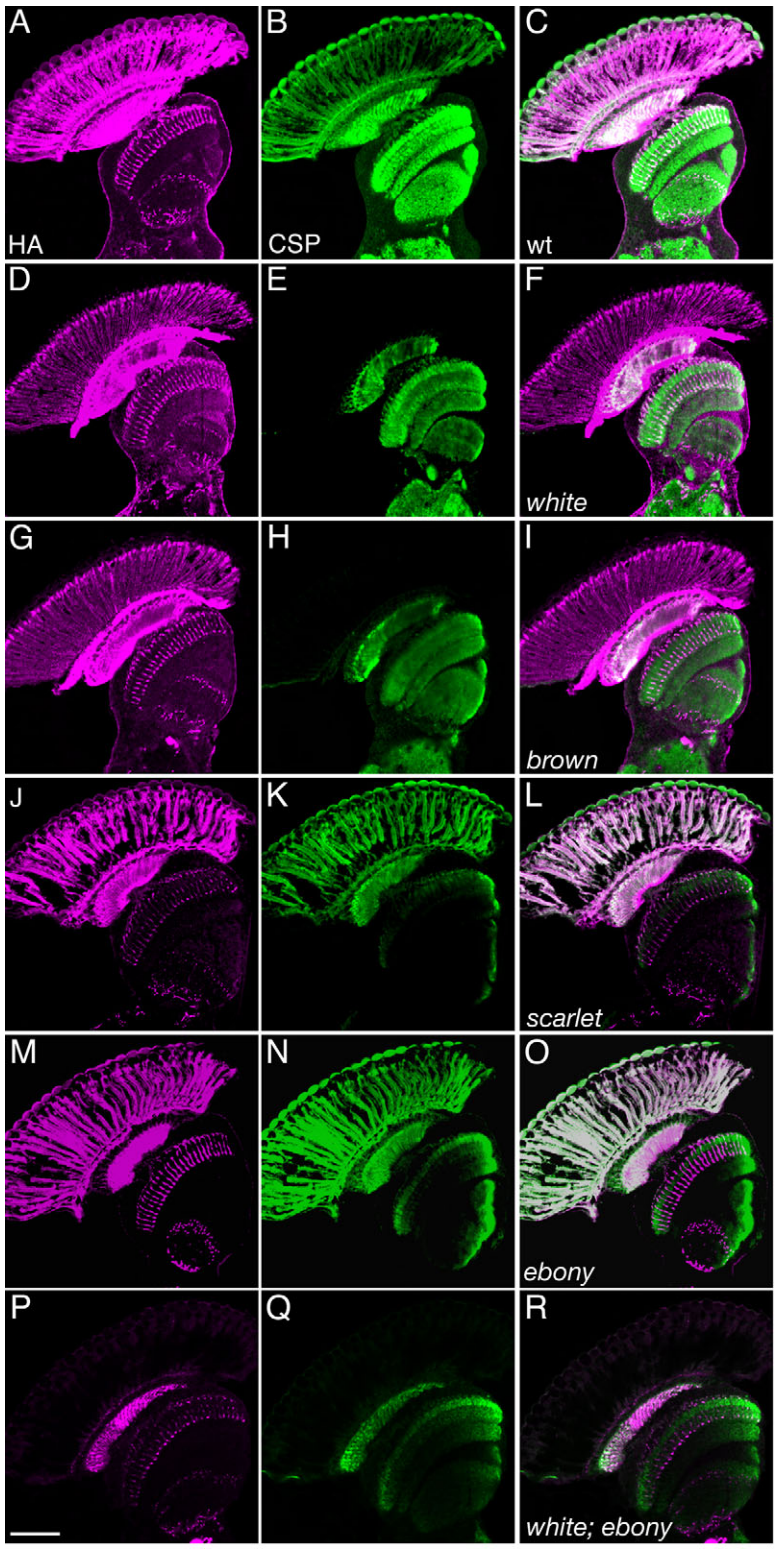

Fig. 2. Immunoreactivity to histamine is reduced in *white*, *brown*, *scarlet*, *ebony* and *white*; *ebony* relative to Oregon-R wild-type flies. Frozen 10 μm frontal sections immunolabeled with anti-CSP (B,E,H,K,N.Q) or anti-histamine (A,D,G,J,M,P), and the corresponding merged double label anti-CSP (green) and anti-histamine (magenta; C,F,I,L,O,R). (A–C) Oregon R wild-type; (D–F) *white*, (G–I) *brown*, (J–L) *scarlet*, (M–O) *ebony* and (P–R) *white*; *ebony*. Relative to the wild-type, the histamine immunosignal is reduced in the laminas of all mutants. The signal is similarly reduced for CSP, in the optic lobe and central brain in *white*, *brown* and largely lost in *white*; *ebony* mutants. Overlap between the expression patterns is complete only in the laminas of wild-type. Scale bar, 100 μm.

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

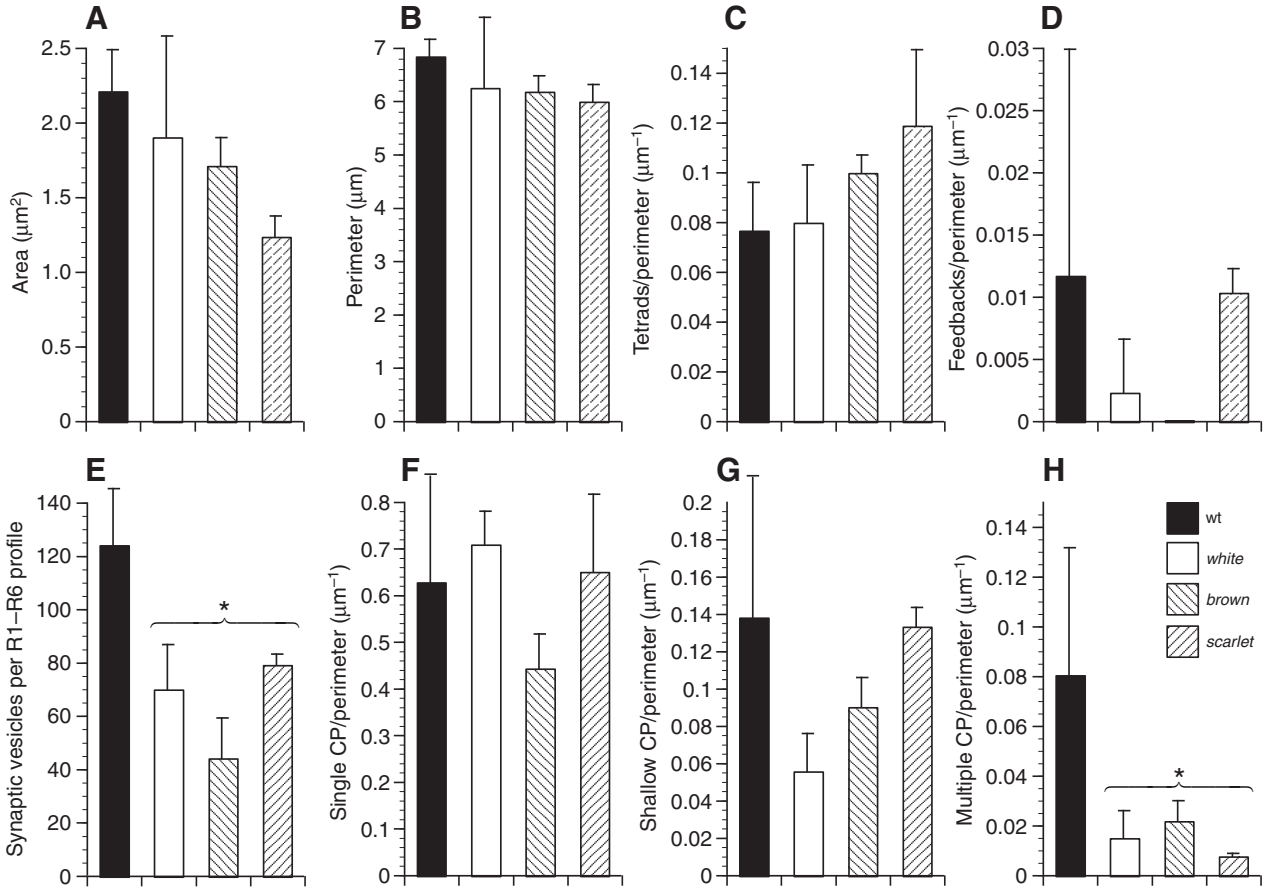

Fig. 3. *white*, *brown* and *scarlet* mutants have organelle counts that mostly do not differ from wild-type controls. All values are mean ± s.d. (N=4 flies). (A,B) Sizes of R1–R6 profiles. Relative to wild-type controls, no differences were detected in profile sizes, either in their cross-sectional 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).

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.

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, are 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

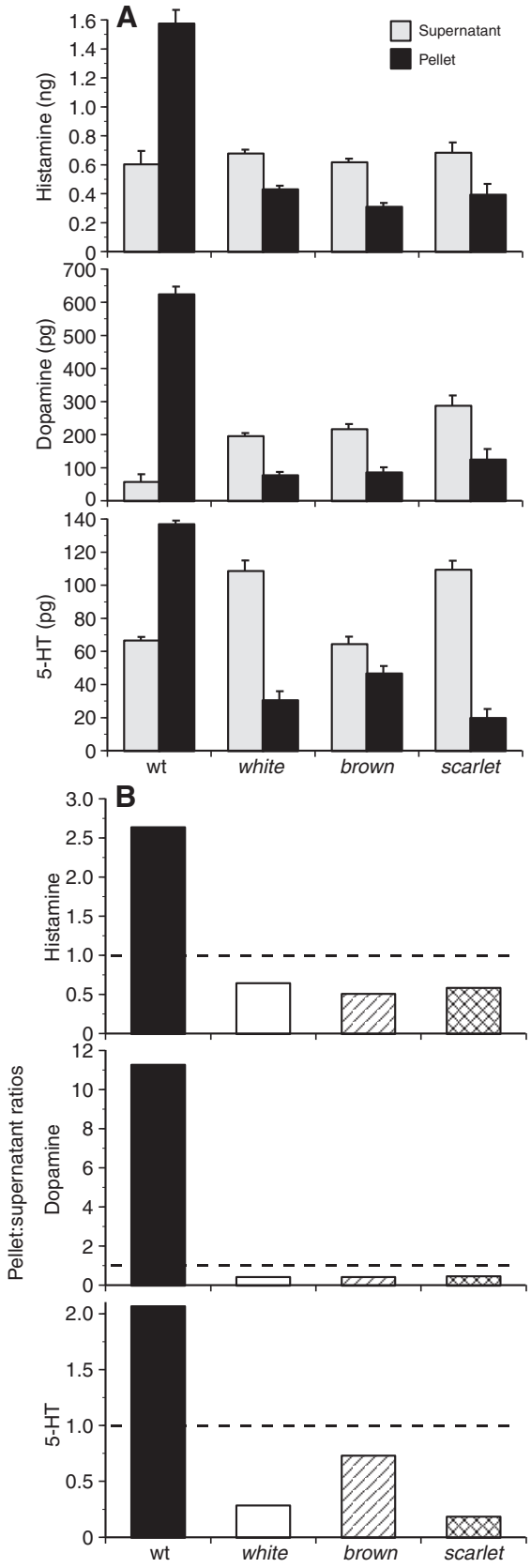

Fig. 4. Vesicle-enriched fractions from *white*, *brown* and *scarlet* mutants have reduced biogenic amines. (A) The pellet and supernatant fractions after centrifuging head homogenates of wild-type and mutant flies, calculated to show the average content per head in ng (histamine) or pg (dopamine, 5-HT). The pellet and supernatant fractions were significantly different (paired *t*-test, $P<0.006$), but the direction of the difference depended on the genotype. Values are means ± s.d. (B) Same data as in A, shown as the corresponding pellet:supernatant ratio for mutant and wild-type fly brain homogenates. These ratios are inverted for the mutant flies, compared with wild-type flies.

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

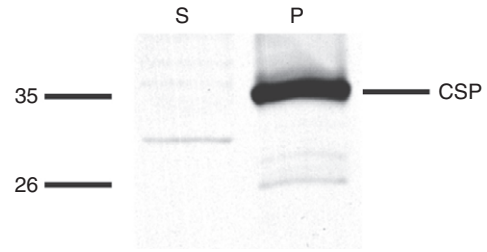

Fig. 5. Western blot of supernatant (S) and pellet (P) fractions from head homogenates probed with anti-cysteine string protein (CSP; see Materials and methods). The antibody detects in the pellet fraction a single band at ~34 kDa, representing the combined isoforms of CSP. This band is not present in the supernatant.

retrieval but also for histamine recycling (Fabian-Fine et al., 2003). 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)

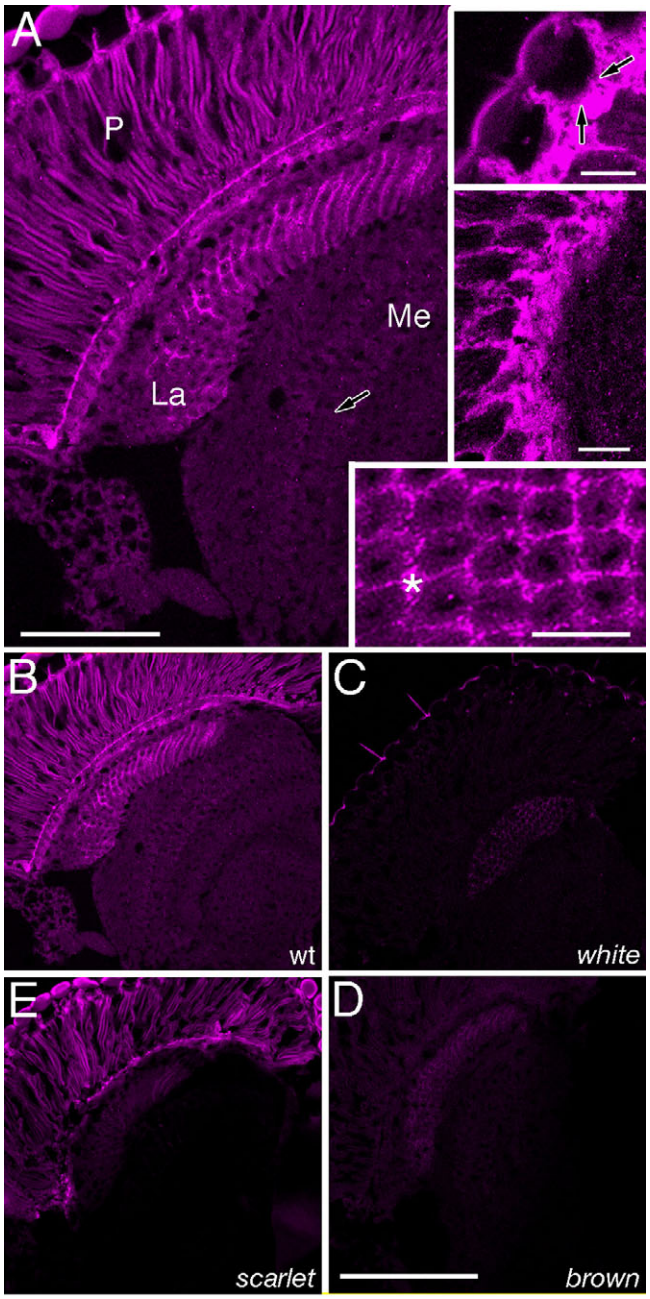

Fig. 6. Immunoreactivity of the *Drosophila* visual system to White protein. Frontal 10 μm cryostat sections of fly heads immunolabeled with a polyclonal antibody that detects the extracellular loop of the White protein. (A) The wild-type shows a strong signal only in the lamina (La), with a much weaker signal in the photoreceptors (P). In the retina, signal is concentrated in the primary pigment cells (inset, top: arrows) and at in the basement membrane (inset, middle), probably in the base of secondary and tertiary pigment cells (Longley and Ready, 1995). In the lamina, White label is clear only in the epithelial glia (inset, bottom: asterisk) that surround the array of lamina cartridges. A ring of R1–R6 terminals is faintly visible within each cartridge, revealing weak photoreceptor expression. Terminals of the R7 and R8 photoreceptors are also faintly visible in the medulla (arrow), but label is otherwise very weak and diffuse in the deeper medulla (Me) neuropile, revealing no cellular expression site. (B–E) Matched wild-type and mutant visual systems immunolabeled in parallel, for comparison. (B) Wild-type. (C) *white* mutant. (D) *brown* mutant. (E) *scarlet* mutant. Relative to the wild-type, the immunosignal is essentially absent in *white* and *brown*, and greatly reduced in *scarlet*. Scale bars, in A, 50 μm and 10 μm (insets); and 100 μm (in D for B–D).

and, as we now see, the epithelial glia in the lamina. Apart from this expression in the periphery of the visual system, the White 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 st^1 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 [^{3}H]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 [^{3}H]histamine. The difference between the [^{3}H]carcinine peaks was significant ($P<0.002$). With *ebony*, greatly reduced [^{3}H]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 [^{3}H]histamine taken up was considerably greater (Fig. 8B), a difference that was significant ($P<0.01$). The greater uptake of [^{3}H]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 [^{3}H]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.

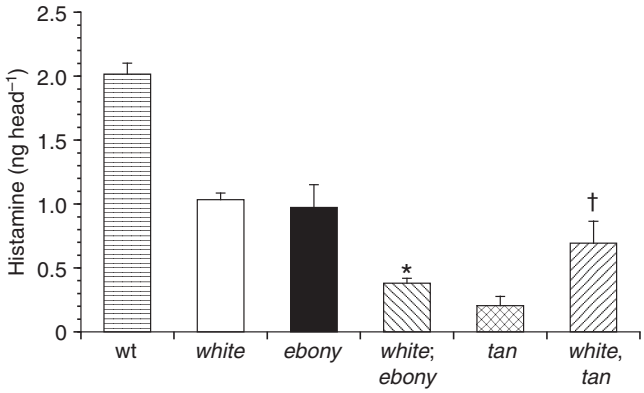

Fig. 7. Histamine in the head is altered reciprocally by *white*, in double mutants with *ebony* and *tan*. In all single mutants, the histamine content is reduced, to 51% (*white*), 48% (*ebony*) and 10% (*tan*) of the wild-type value. Relative to these, double-mutant *white*, *tan* has significantly 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 (*$P<0.0005$).

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 ($P \ll 10^{-6}$ 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

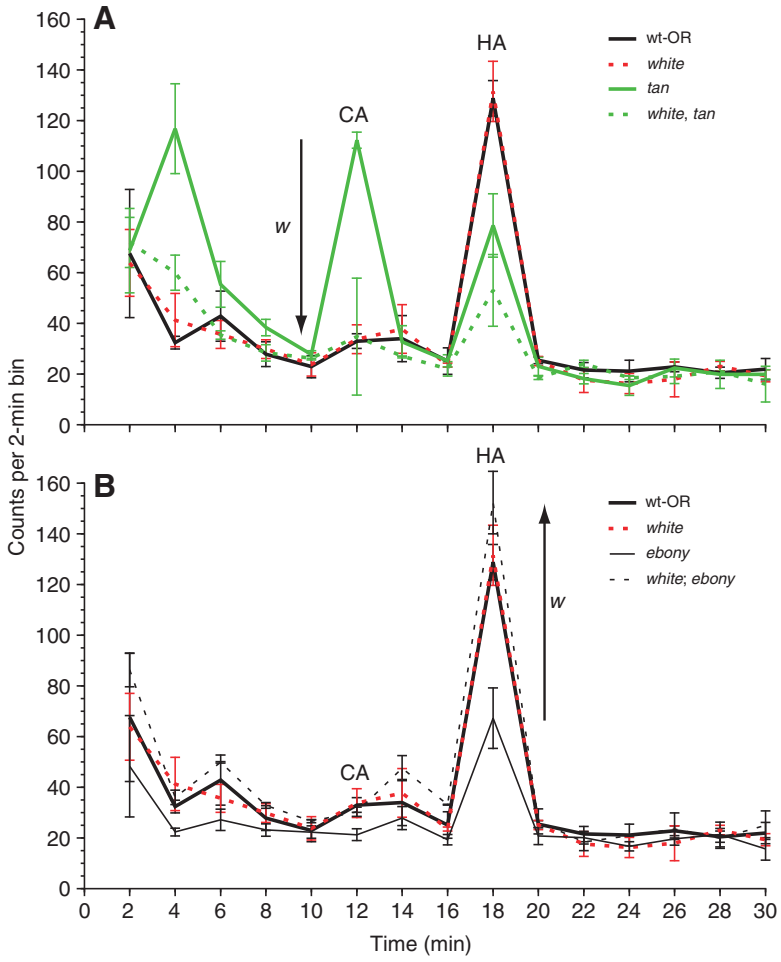

Fig. 8. HPLC separation of head homogenates from flies that drank a 25% solution of [^{3}H]histamine (37 MBq ml^{-1} and 858.4 GBq mmol l^{-1}) in 4% aqueous glucose. (A) Wild-type, *tan* and double-mutant *white*, *tan* heads. (B) Wild-type, *ebony* and double-mutant *white*; *ebony* heads. Double-mutant *white*, *tan* has a significantly reduced carcinine (CA) peak compared with *tan* whereas double-mutant *white*; *ebony* rescues normal histamine (HA) uptake, which is otherwise significantly reduced in *ebony*. Both differences are indicated by arrows delimiting the magnitude of *white*'s (*w*) added action. All data are plotted as 2-min bins, compiled from the emissions from two adjacent 1-min fractions. Retention time of carcinine is 12 min and retention time of histamine is 18 min. The *tan* peak with the shorter retention time (4 min) is an unknown metabolite. For a detailed comment see Borycz et al. (Borycz et al., 2000).

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 *Ddc*-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

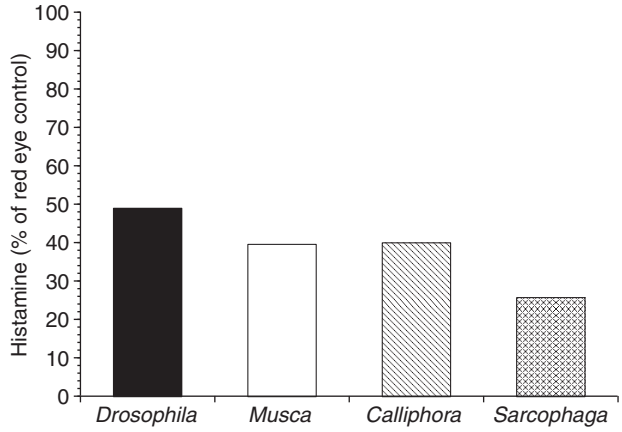

Fig. 9. Histamine is reduced in the heads of four fly species with white eyes. These are: *Sarcophaga* spp., combined data from two species that compare *S. bullata* (wild-type) with *S. barbata* (mutant ivory, with white eyes); *Calliphora erythrocephala* (wild-type) with *chalky* (white eye); *Musca domestica* (wild-type and white-eyed); and *Drosophila melanogaster*, *white* null mutant and O-R wild-type.

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 *ebony* 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 (Faúndez 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 *ebony* (Richardt 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*

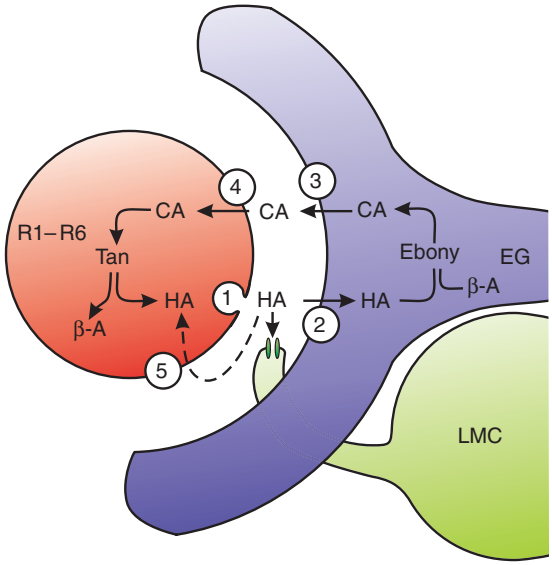

Fig. 10. White's proposed involvement in the *ebony–tan* histamine shuttle. Histamine (HA) is released from the photoreceptor terminal (R1–R6) into the synaptic cleft (1), where it activates postsynaptic histamine receptors on the dendrites of a lamina monopolar cell (LMC) target. Its action is terminated by glial uptake (2), which our evidence indicates may be partly *white* dependent. Histamine in the epithelial glia (EG) is then conjugated to β-alanine (β-A) to form carcinine (CA), regulated by Ebony, and the carcinine then extruded from the glia by an unknown transporter (3). Carcinine is next taken up from the cleft by the transporter Inebriated (4) (Gavin et al., 2007), into the R1–R6 terminal, where it is hydrolyzed under the action of Tan to liberate histamine and β-alanine. No clear evidence exists for a direct histamine reuptake mechanism at the R1–R6 terminal membrane (5).

oculis mutants, which lacks compound eyes (Campbell and Nash, 2001). Most likely, therefore, transcriptional levels in the brain are too low to detect.

The possibility of a more general effect of *white* in cells other than the visual system and for other amines than histamine, is hard to address. Glial cells are very slender and enwrapping and lack endosomes that are easily detected, and possible storage sites in alternative neurons that might use other biogenic amines are equally inaccessible. This is why we have studied the most accessible neurons in one of the best-characterized neuropiles of the fly's brain, which also has the largest amount of any amine. All other systems pose much less favorable alternatives.

A role for *white* in the lamina

Although the outcome of *white*'s action may lie in a partial loss of intravesicular bioamine, at least in the visual system this action is indirect, and occurs *via* epithelial glial expression that must affect histamine recycling through the photoreceptor-glial shuttle (Stuart et al., 2007). *Tan* mutants accumulate carcinine, which they synthesize but cannot hydrolyze (Borycz et al., 2002), and so show a large peak of [^{3}H]carcinine, whereas double-mutant *white*, *tan* convert less [^{3}H]histamine to [^{3}H]carcinine than do *tan* single mutants (Fig. 8A). This decrease is consistent with reduced [^{3}H]histamine uptake by the epithelial glia, and we therefore consider a tentative model in which histamine uptake by the epithelial glia is *white* dependent. *ebony* mutants fail to trap [^{3}H]histamine as carcinine, which they cannot synthesize (Borycz et al., 2002), and thus have no way to retain ingested tritium, thus having less [^{3}H]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 [^{3}H]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.

This work was supported by a NATO fellowship (to J.B.), by NSERC grant D6307 (to V.L.) and by NIH grant EY-03592 and CIHR grant ROP-6740 (to I.A.M.). We thank Ms Rita Kostyleva for assistance with electron microscopy, Dr Konrad Zinsmaier (University of Arizona, Tucson, AZ, USA) for advice on monoclonal 49-1 anti-CSP and Dr Gary Ewart (Australian National University, Canberra) for providing the White antibody.

REFERENCES

Ames, G. F. L. (1986). The basis of multidrug resistance in mammalian cells: homology with bacterial transport. *Cell* **47**, 323-324.
Borycz, J., Borycz, J. A., Loubani, M. and Meinertzhagen, I. A. (2002). *tan* and *ebony* genes regulate a novel pathway for transmitter metabolism at fly photoreceptor terminals. *J. Neurosci.* **22**, 10549-10557.
Borycz, J. A., Borycz, J., Kostyleva, R. and Meinertzhagen, I. A. (2005a). *Drosophila* ABC transporter mutants *white*, *scarlet* and *brown* have an altered head content and distribution of biogenic amines. *Abstr. Soc. Neurosci.* **31**, 30.16.
Borycz, J. A., Borycz, J., Kubów, A., Kostyleva, R. and Meinertzhagen, I. A. (2005b). Histamine compartments of the *Drosophila* brain with an estimate of the quantum content at the photoreceptor synapse. *J. Neurophysiol.* **93**, 1611-1619.
Borycz, J., Vohra, M., Tokarczyk, G. and Meinertzhagen, I. A. (2000). The determination of histamine in the *Drosophila* head. *J. Neurosci. Methods* **101**, 141-148.
Bronk, P., Nie, Z., Klose, M. K., Dawson-Scully, K., Zhang, J., Robertson, R. M., Atwood, H. L. and Zinsmaier, K. E. (2005). The multiple functions of cysteine-string protein analyzed at *Drosophila* nerve terminals. *J. Neurosci.* **25**, 2204-2214.
Campbell, J. L. and Nash, H. A. (2001). Volatile general anesthetics reveal a neurobiological role for the *white* and *brown* genes of *Drosophila melanogaster*. *J. Neurobiol.* **49**, 339-349.
Dawson-Scully, K., Bronk, P., Atwood, H. L. and Zinsmaier, K. E. (2000). Cysteine-string protein increases the calcium sensitivity of neurotransmitter exocytosis in *Drosophila*. *J. Neurosci.* **20**, 6039-6047.
Diegelmann, S., Zars, M. and Zars, T. (2006). Genetic dissociation of acquisition and memory strength in the heat-box spatial learning paradigm in *Drosophila*. *Learn. Mem.* **13**, 72-83.

Dierick, H. A. and Greenspan, R. J. (2007). Serotonin and neuropeptide F have opposite modulatory effects on fly aggression. *Nat. Genet.* **39**, 678-682.
Dräger, U. C. and Balkema, G. W. (1987). Does melanin do more than protect from light? *Neurosci. Res.* **Suppl. 6**, S75-S86.
Dreesen, T. D., Johnson, D. H. and Henikoff, S. (1988). The brown protein of *Drosophila melanogaster* is similar to the white protein and to components of active transport complexes. *Mol. Cell. Biol.* **8**, 5206-5215.
Eberle, K. K., Zinsmaier, K. E., Buchner, S., Gruhn, M., Jenni, M., Arnold, C., Leibold, C., Reisch, D., Walter, N., Hafen, E. et al. (1998). Wide distribution of the cysteine string proteins in *Drosophila* tissues revealed by targeted mutagenesis. *Cell Tissue Res.* **294**, 203-217.
Ewart, G. D. and Howells, A. J. (1998). ABC transporters involved in transport of eye pigment precursors in *Drosophila melanogaster. Meth. Enzymol.* **292**, 213-224.
Ewart, G. D., Cannell, D., Cox, G. B. and Howells, A. J. (1994). Mutational analysis of the traffic ATPase (ABC) transporters involved in uptake of eye pigment precursors in *Drosophila melanogaster. J. Biol. Chem.* **269**, 10370-10377.
Fabian-Fine, R., Verstreken, P., Hiesinger, P. R., Horne, J. A., Kostyleva, R., Bellen, H. J. and Meinertzhagen, I. A. (2003). Endophilin acts after synaptic vesicle fission in *Drosophila* photoreceptor terminals. *J. Neurosci.* **23**, 10732-10744.
Faúndez, V., Horng, J. T. and Kelly, R. B. (1998). A function for the AP3 coat complex in synaptic vesicle formation from endosomes. *Cell* **93**, 423-432.
Fjose, A., Polito, L. C., Weber, U. and Gehring, W. J. (1984). Developmental expression of the *white* locus of *Drosophila melanogaster. EMBO J.* **3**, 2087-2094.
Fujita, S. C., Inoue, H., Yoshioka, T. and Hotta, Y. (1987). Quantitative tissue isolation from *Drosophila* freeze-dried in acetone. *Biochem. J.* **243**, 97-104.
Gavin, B. A., Arruda, S. E. and Dolph, P. J. (2007). The role of carcinine in signaling at the Drosophila photoreceptor synapse. *PLOS Genetics* **3**, e206.
Geffard, M., Buijs, R. M., Seguela, P., Pool, C. W. and Le Moal, M. (1984). First demonstration of highly specific and sensitive antibodies against dopamine. *Brain Res.* **294**, 161-165.
Guillery, R. W. (1986). Neural abnormalities of albinos. *Trends Neurosci.* **9**, 364-367.
Hadorn, E. and Mitchell, H. K. (1951). Properties of mutants of *Drosophila melanogaster* and changes during development as revealed by paper chromatography. *Proc. Natl. Acad. Sci. USA* **37**, 650-665.
Hardie, R. C. (1987). Is histamine a neurotransmitter in insect photoreceptors? *J. Comp. Physiol. A* **161**, 201-213.
Hardie, S. L. and Hirsh, J. (2006). An improved method for separation and detection of biogenic amines in adult *Drosophila* brain extracts by high performance liquid chromatography. *J. Neurosci. Methods* **153**, 243-249.
Hengstenberg, R. and Götz, K. G. (1967). Der Einfluss des Schrimpigmentgehalts auf die Helligkeits- und Kontrastwahrnehmung bei *Drosophila*-Augenmutanten. *Kybernetik* **3**, 276-285.
Hiesinger, P. R., Zhai, R. G., Zhou, Y., Koh, T.-W., Mehta, S. Q., Schulze, K. L., Cao, Y., Verstreken, P., Clandinin, T. R., Fischbach, K.-F. et al. (2006). Activity-independent prespecification of synaptic partners in the visual map of *Drosophila*. *Curr. Biol.* **16**, 1835-1843.
Higgins, C. F. (1992). ABC transporters – from microorganisms to man. *Annu. Rev. Cell Biol.* **8**, 67-113.
Hing, A. L. and Carlson, J. R. (1996). Male-male courtship behavior induced by ectopic expression of the *Drosophila white* gene: role of sensory function and age. *J. Neurobiol.* **30**, 454-464.
Jeffery, G. (1997). The albino retina: an abnormality that provides insight into normal retinal development. *Trends Neurosci.* **20**, 165-169.
Kalmus, H. (1943). The optomotor responses of some eye mutants in *Drosophila. J. Genet.* **45**, 206-213.
Li, H., Chaney, S., Forte, M. and Hirsh, J. (2000). Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in *Drosophila melanogaster Curr. Biol.* **10**, 211-214.
Lloyd, V. K., Sinclair, D. A., Alperyn, M. and Grigliatti, T. A. (2002). Enhancer of garnet/deltaAP-3 is a cryptic allele of the *white* gene and identifies the intracellular transport system for the white protein. *Genome* **45**, 296-312.
Longley, R. L. and Ready, D. F. (1995). Integrins and the development of three-dimensional structure in the Drosophila compound eye. *Dev. Biol.* **171**, 415-433.
Mackenzie, S. M., Howells, A. J., Cox, G. B. and Ewart, G. D. (2000). Sub-cellular localisation of the white/scarlet ABC transporter to pigment granule membranes within the compound eye of *Drosophila melanogaster. Genetica* **108**, 239-252.
Meinertzhagen, I. A. (1996). Ultrastructure and quantification of synapses in the insect nervous system. *J. Neurosci. Methods* **69**, 59-73.
Meinertzhagen, I. A. and O'Neil, S. D. (1991). Synaptic organization of columnar elements in the lamina of the wild type in *Drosophila melanogaster. J. Comp. Neurol.* **305**, 232-263.
Meinertzhagen, I. A. and Sorra, K. E. (2001). Synaptic organisation in the fly's optic lamina: few cells, many synapses and divergent microcircuits. *Prog. Brain Res.* **131**, 53-69.
Melzig, J., Buchner, S., Wiebel, F., Wolf, R., Burg, M., Pak, W. L. and Buchner, E. (1996). Genetic depletion of histamine from the nervous system of *Drosophila* eliminates specific visual and mechanosensory behavior. *J. Comp. Physiol. A* **179**, 763-773.
Morgan, T. H. (1910). Sex limited inheritance in *Drosophila. Science* **32**, 120-122.
Mount, S. M. (1987). Sequence similarity. *Nature* **325**, 487.
Nässel, D. R. (1987). Serotonin and serotonin-immunoreactive neurons in the nervous system of insects. *Prog. Neurobiol.* **30**, 1-85.
Nässel, D. R., Meyer, E. P. and Klemm, N. (1985). Mapping and ultrastructure of serotonin-immunoreactive neurons in the optic lobes of three insect species. *J. Comp. Neurol.* **232**, 190-204.
Nässel, D. R., Elekes, K. and Johansson, K. U. I. (1988). Dopamine-immunoreactive neurons in the blowfly visual system: light and electron microscopic immunocytochemistry. *J. Chem. Neuroanat.* **1**, 311-325.
Neckameyer, W., Woodrome, S., Holt, B. and Mayer, A. (2000). Dopamine and senescence in *Drosophila melanogaster. Neurobiol. Aging* **21**, 145-152.
Newsome, T. P., Åsling, B. and Dickson, B. J. (2000). Analysis of *Drosophila* photoreceptor axon guidance in eye-specific mosaics. *Development* **127**, 851-860.
Oyster, C. W. (1999). *The Human Eye: Structure and Function.* Sunderland, MA: Sinauer.
Pak, W. L., Grossfield, J. and White, N. V. (1969). Nonphototactic mutants in a study of vision of *Drosophila. Nature* **222**, 351-354.
Parsons, S. M., Prior, C. and Marshall, I. G. (1993). Acetylcholine transport, storage, and release. *Int. Rev. Neurobiol.* **35**, 279-390.
Phillips, J. P. and Forrest, H. S. (1980). Ommochromes and pteridines. In *The Genetics and Biology of Drosophila*. Vol. 2 (ed. M. Ashburner and T. R. F. Wright), pp. 541-623. New York: Academic Press.
Pollack, I. and Hofbauer, A. (1991). Histamine-like immunoreactivity in the visual system and brain of *Drosophila melanogaster. Cell Tissue Res.* **266**, 391-398.
Pyza, E. and Meinertzhagen, I. A. (1997). Circadian rhythms in screening pigment and invaginating organelles in photoreceptor terminals of the housefly's first optic neuropile. *J. Neurobiol.* **32**, 517-529.
Pyza, E. and Meinertzhagen, I. A. (1998). Neurotransmitters alter the numbers of synapses and organelles in photoreceptor terminals in the lamina of the housefly, *Musca domestica. J. Comp. Physiol. A* **183**, 719-727.
Regnier-Vigouroux, A. and Huttner, W. B. (1993). Biogenesis of small synaptic vesicles and synaptic-like microvesicles. *Neurochem. Res.* **18**, 59-64.
Richardt, A., Rybak, J., Störtkuhl, K. F., Meinertzhagen, I. A. and Hovemann, B. T. (2002). Ebony protein in the *Drosophila* nervous system: optic neuropile expression in glial cells. *J. Comp. Neurol.* **452**, 93-102.
Richardt, A., Kemme, T., Wagner, S., Schwarzer, D., Marahiel, M. A. and Hovemann, B. T. (2003). Ebony, a novel nonribosomal peptide synthetase for beta-alanine conjugation with biogenic amines in *Drosophila. J. Biol. Chem.* **278**, 41160-41166.
Sang, T.-K., Chang, H.-Y., Lawless, G. M., Ratnaparkhi, A., Mee, L., Ackerson, L. C., Maidment, N. T., Krantz, D. E. and Jackson, G. R. (2007). A *Drosophila* model of human parkin-induced toxicity demonstrates selective loss of dopaminergic neurons and dependence on cellular dopamine. *J. Neurosci.* **27**, 981-992.
Savvateeva, E. V., Popov, A. V., Kamyshev, N. G., Iliadi, K. G., Bragina, J. V., Heisenberg, M., Kornhuber, J. and Riederer, P. (1999). Age-dependent changes in memory and mushroom bodies in the *Drosophila* mutant *vermilion* deficient in the kynurenine pathway of tryptophan metabolism. *Ross. Fiziol. Zh. Im I. M. Sechenova* **85**, 167-183.
Savvateeva, E., Popov, A., Kamyshev, N., Bragina, J., Heisenberg, M., Senitz, D., Kornhuber, J. and Riederer, P. (2000). Age-dependent memory loss, synaptic pathology and altered brain plasticity in the *Drosophila* mutant *cardinal* accumulating 3-hydroxykynurenine. *J. Neural Transm.* **107**, 581-601.
Shoup, J. R. (1966). The development of pigment granules in the eyes of wild type and mutant *Drosophila melanogaster. J. Cell Biol.* **29**, 223-249.
Stark, W. S. and Carlson, S. D. (1986). Ultrastructure of capitate projections in the optic neuropil of Diptera. *Cell Tiss. Res.* **246**, 481-486.
Stowers, R. S. and Schwarz, T. L. (1999). A genetic method for generating *Drosophila* eyes composed exclusively of mitotic clones of a single genotype. *Genetics* **152**, 1631-1639.
Stuart, A. E., Borycz, J. and Meinertzhagen, I. A. (2007). The dynamics of signaling at the histaminergic photoreceptor synapse of arthropods. *Prog. Neurobiol.* **82**, 202-227.
Sullivan, D. T. and Sullivan, M. C. (1975). Transport defects as the physiological basis for eye color mutants of *Drosophila melanogaster. Biochem. Genet.* **13**, 603-613.
Sullivan, D. T., Bell, L. A., Paton, D. R. and Sullivan, M. C. (1979). Purine transport by malpighian tubules of pteridine-deficient eye color mutants of *Drosophila melanogaster. Biochem. Genet.* **17**, 565-573.
Summers, K. M., Howells, A. J. and Pyliotis, N. A. (1982). Biology of eye pigmentation in insects. *Adv. Insect Physiol.* **16**, 119-166.
Tabb, J. S. and Ueda, T. (1991). Phylogenetic studies on the synaptic vesicle glutamate transport system. *J. Neurosci.* **11**, 1822-1828.
True, J. R., Yeh, S.-D., Hovemann, B., Kemme, T., Meinertzhagen, I. A., Edwards, T., Liou, S.-R., Li, J. (2005). *Drosophila tan* encodes a novel N -alanyl dopamine/ -alanyl histamine hydrolase required for melanin pigmentation and photoreceptor neurotransmitter metabolism. *PloS Genetics* **1**, 551-562.
Vallés, A. M. and White, K. (1986). Development of serotonin-containing neurons in *Drosophila* mutants unable to synthesize serotonin. *J. Neurosci.* **6**, 1482-1491.
van de Goor, J. and Kelly, R. B. (1996). Association of *Drosophila* cysteine string proteins with membranes. *FEBS Lett.* **380**, 251-256.
van de Goor, J., Ramaswami, M. and Kelly, R. B. (1995). Redistribution of synaptic vesicles and their proteins in temperature-sensitive *shibire*^{ts1} mutant *Drosophila*. *Proc. Natl. Acad. Sci. USA* **92**, 5739-5743.
Wagner, S., Heseding, C., Szlachta, K., True, J. R., Prinz, H. and Hovemann, B. T. (2007). *Drosophila* photoreceptors express cysteine peptidase *tan. J. Comp. Neurol.* **500**, 601-611.
Wittkopp, P. J., True, J. R. and Carroll, S. B. (2002). Reciprocal functions of the *Drosophila* Yellow and Ebony proteins in the development and evolution of pigment patterns. *Development* **129**, 1849-1858.
Wu, C.-F. and Wong, F. (1977). Frequency characteristics in the visual system of *Drosophila. J. Gen. Physiol.* **69**, 705-724.
Zhang, S.-D. and Odenwald, W. F. (1995). Misexpression of the *white* (*w*) gene triggers male-male courtship in *Drosophila. Proc. Natl. Acad. Sci. USA* **92**, 5525-5529.
Zinsmaier, K. E., Hofbauer, A., Heimbeck, G., Pflugfelder, G. O., Buchner, S. and Buchner, E. (1990). A cysteine-string protein is expressed in retina and brain of *Drosophila. J. Neurogenet.* **7**, 15-29.
Zinsmaier, K. E., Eberle, K. K., Buchner, E., Walter, N. and Benzer, S. (1994). Paralysis and early death in cysteine string protein mutants of *Drosophila. Science* **263**, 977-980.