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RESEARCH ARTICLE

The metabolism of histamine in the *Drosophila* optic lobe involves an ommatidial pathway: β-alanine recycles through the retina

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SUMMARY

Flies recycle the photoreceptor neurotransmitter histamine by conjugating it to β-alanine to form β-alanyl-histamine (carcinine). The conjugation is regulated by Ebony, while Tan hydrolyses carcinine, releasing histamine and β-alanine. In *Drosophila*, β-alanine synthesis occurs either from uracil or from the decarboxylation of aspartate but detailed roles for the enzymes responsible remain unclear. Immunohistochemically detected β-alanine is present throughout the fly's entire brain, and is enhanced in the retina especially in the pseudocone, pigment and photoreceptor cells of the ommatidia. HPLC determinations reveal 10.7 ng of β-alanine in the wild-type head, roughly five times more than histamine. When wild-type flies drink uracil their head β-alanine increases more than after drinking L-aspartic acid, indicating the effectiveness of the uracil pathway. Mutants of *black*, which lack aspartate decarboxylase, cannot synthesize β-alanine from L-aspartate but can still synthesize it efficiently from uracil. Our findings demonstrate a novel function for pigment cells, which not only screen ommatidia from stray light but also store and transport β-alanine and carcinine. This role is consistent with a β-alanine-dependent histamine recycling pathway occurring not only in the photoreceptor terminals in the lamina neuropile, where carcinine occurs in marginal glia, but vertically *via* a long pathway that involves the retina. The lamina's marginal glia are also a hub involved in the storage and/or disposal of carcinine and β-alanine.

Key words: fly, photoreceptors, carcinine, glia, lamina, cone cell, pigment cell.

INTRODUCTION

A hundred years after its discovery, the possible role in the nervous system of β-alanine, a β-amino acid structurally similar to γ-aminobutyric acid (GABA) and glycine, remains a mystery. Its ubiquity, its multiple pathways of biosynthesis and the pleiotropy of genes regulating those pathways are all factors that continue to frustrate studies on β-alanine. Early studies indicated that uracil, aspartate, panthothenate and propionate are all precursors of β-alanine in insects (Jacobs, 1968; Ross and Monroe, 1972), highlighting the difficulties in analysing the pathways for β-alanine biosynthesis.

In insects, β-alanine is required for cuticle melanization (Jacobs, 1980; Wright, 1987) and for vision. In the visual system, histamine – a neurotransmitter released by the synaptic terminals of fly photoreceptor neurons (Hardie, 1987; Sarthy, 1991) – is inactivated by conjugation to β-alanine under the action of the β-alanyl biogenic amine synthetase Ebony, which is expressed in neighbouring epithelial glial cells (Borycz et al., 2002). Ebony also acts in a similar fashion on other biogenic amines in the brain of the fruit fly *Drosophila melanogaster* (Richardt et al., 2003), confirming an early suggestion that β-alanylation may be a general pathway for inactivating biogenic amines in insects (Evans, 1980; Blenau and Baumann, 2005).

The two most common pathways for the synthesis of β-alanine are from aspartate and from uracil (Fig. 1). An alternative source for β-alanine is sarcophagine (β-alanyl-L-tyrosine), as suggested by Hodgetts (Hodgetts, 1972). The biosynthesis of β-alanine by decarboxylation of L-aspartate is regulated by an aspartate decarboxylase that in *Drosophila* is encoded by the gene *black* (Hodgetts, 1972). This is expressed along with *ebony* in the epithelial glial cells of the first optic neuropile, or lamina, that surround the terminals of photoreceptor neurons R1–R6 (Phillips et al., 2005). The strength and location of its expression have understandably focused attention on Black as the main regulator for the supply of β-alanine. Uracil is an alternative source of β-alanine, however, and is metabolized by the consecutive actions of three enzymes: dihydropyrimidine dehydrogenase (Pyd1), dihydropyrimidine amidohydrolase (Pyd2) and β-ureidopropionase (Pyd3) (Fig. 1C) (Piskur et al., 2007). The last of these, β-ureidopropionase or β-alanine synthase (Matthews and Traut, 1987), is the sole enzyme catalysing the synthesis of β-alanine from uracil, and differs from the β-alanine synthase of prokaryotes (Andersen et al., 2008) but is similar to mammalian *pyd3* (Rawls, 2006). In *Drosophila*, the crystal structure of Pyd3 has been reported (Lundgren et al., 2008) and mutants isolated (Rawls, 2006). Mutants for the genes that encode enzymes regulating these different biosynthetic pathways in *Drosophila* can be used in instrumental approaches that manipulate β-alanine production, but knowledge of these several pathways is required to interpret experiments in which one or other is blocked or altered.

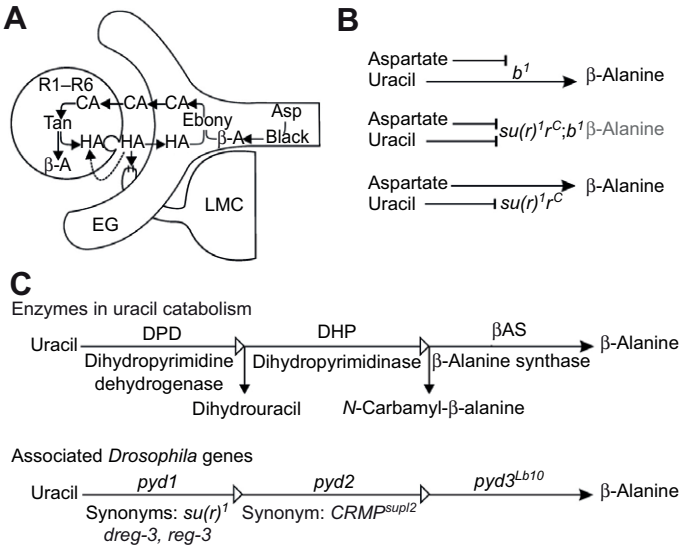

Fig. 1. Pathways for the biosynthesis and recycling of β-alanine. (A) After its release from the photoreceptor terminal, where it acts on the dendrites of large monopolar cells (LMC), histamine (HA) is recycled by a glial shuttle pathway in the lamina that requires β-alanine (β-A) in the epithelial glia (EG) for the biosynthesis of β-alanyl-histamine (carcinine, CA) under the influence of Ebony, and that releases β-alanine along with histamine in the photoreceptor terminal (R1–R6) under the action of Tan. (B) In insects, β-alanine is synthesized from aspartate or uracil by two main pathways, regulated either from aspartate by aspartate decarboxylase (*black*), or from uracil (C) in succession by dihydropyrimidine dehydrogenase [Pyd1, encoded by *su(r)*^{1}], dihydropyrimidine amidohydrolase (dihydropyrimidase, Pyd2) and β-ureidopropionase (β-alanine synthase, Pyd3). Synonyms for the corresponding *Drosophila* genes involved in the synthesis of β-alanine from uracil are indicated. Mutant *black*^{1} (*b*^{1}) lacks the first pathway, mutant *su(r)*^{1}*r*^{C} lacks the second pathway and double mutant *su(r)*^{1}*r*^{C}; *b*^{1} lacks both pathways (B).

Not only is knowledge lacking on the particular biochemical pathways used to recycle β-alanine but also the cellular pathways for that recycling have not been identified. The visual system offers the clearest substrate for such identification, because its constituent cell types are all clearly identified, not only in the retina (Ready et al., 1976) but also in the underlying lamina, both neurons (Meinertzhagen and O'Neil, 1991) and glia (Edwards and Meinertzhagen, 2010). Here, we dissected the roles of the two main pathways for the β-alanine synthesis that is required to recycle histamine in the visual system. We show that both contribute to the brain's β-alanine pool and that new synthesis rather than recycling is the main source of the amino acid. We reveal that β-alanine is stored in high concentrations in ommatidia, cone cells and also primary and secondary pigment cells.

MATERIALS AND METHODS

Animals

Flies, *Drosophila melanogaster* Meigen, were held at 23°C on a standard cornmeal and molasses medium under a 12 h:12 h light:dark cycle. The following genotypes were used: (1) Oregon R (OR) wild-type; (2) *b*^{1} *(black*^{1}*)*; (3) triple mutant *su(r)*^{1}*r*^{C}; *b*^{1} (*suppressor of rudimentary*, *rudimentary* and *black*); (4) double mutant *su(r)*^{1}*r*^{C}; (5) *w**; *b**; *CRMP*^{supl2}*/TM3*, *Sb*^{1} *Ser*^{1}; (6) *w**; *b*^{1}; *pyd3*^{Lb10}*/TM3*, *Sb*^{1} *Ser*^{1}; (7) *w**; *b*^{1}; *pyd3*^{Lb5}*/TM3*, *Sb*^{1} *Ser*^{1}; (8) *w**; *P{PYD3+}3b b*^{1}; *pyd3*^{Lb10}*/TM3*, *Sb*^{1} *Ser*^{1}. Of these, 3 and 4 are mutants of *pyd1*; 5 is a mutant of *pyd2*, a dihydropyrimidinase (CRMP) gene; 6 and 7 are double mutants of *black* and *pyd3*, a β-alanine synthase gene; and 8 is a rescue of the *pyd3* mutant (Rawls, 2006). We also used two reporter lines, *spa-lacZ* (a gift of Dr Utpal Banerjee, UCLA, USA) and *rh1*(–252/+67)-*lacZ*.omSMB (Mismer and Rubin, 1987), referred to as *rh1-lacZ*. We also used *tan*^{1} and two *ebony* alleles, *ebony*^{1} and *In(3R)e*^{AFA}.

To administer reagents, flies were dehydrated for 3 h and then given a drink of 4% aqueous solution of glucose laced with L-aspartic acid, uracil or diaminobutyric acid (DABA), as previously reported (Borycz et al., 2002; Borycz et al., 2008).

High-performance liquid chromatography (HPLC)

For determinations of brain β-alanine and histamine, flies were collected, frozen at –80°C, shaken to decapitate them, and the heads then processed using HPLC with electrochemical detection, all as previously reported (Borycz et al., 2000). In this method, β-alanine is clearly separated from neighbouring peaks and has a retention time that is approximately 2 min shorter than that for histamine. Sometimes, to obtain better resolution of the β-alanine peak, the acetonitrile content in the mobile phase was adjusted by about 10%. We used samples from 50 *Drosophila* heads, and calculated the mean of the mean values from between 7 and 12 samples.

Uptake of tritiated β-alanine

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]β-alanine (37 MBq ml^{-1} and 1850 GBq mmol l^{-1}; American Radiolabeled Chemicals Inc., St Louis, MO, USA) in 4% aqueous glucose to drink. After 40 min, the 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, Mississauga, ON, Canada), were counted for 5 min in a scintillation counter (Beckman Coulter LS 6500). The retention time for [^{3}H]β-alanine and its metabolite tritiated-β-alanyl-histamine ([^{3}H]carcinine) in these fractions was confirmed exactly from the retention time for the histamine and carcinine peaks obtained by electrochemical detection.

Electroretinogram (ERG) recordings

Female flies were first immobilized on ice and then secured in the cut end of a 10 µl pipette tip using nail polish. A silver reference electrode was placed in the abdomen and a 1.2 mm glass and silver recording electrode filled with *Drosophila* Ringer solution was placed on the surface of the cornea. Flies were exposed to 300 ms light pulses from a blue light-emitting diode (LEDtronics type BP120CWPB2K-300, Torrance, CA, USA) driven by a Grass SD9 stimulator (Grass Technologies, West Warwick, RI, USA) at 3.5 V. For each fly, a 2 min period of dark adaptation was followed by four series of 10 recordings, and each series averaged using A/dvance P 3.61g software (Dr R. M. Douglas, McKellar Designs UBC, Vancouver, BC, Canada). A series comprised 10 pulses separated by a 20 s dark interval.

Statistical analysis

Throughout, values of biogenic amines are expressed as the mean ± s.d. of the mean values for 7–12 independent samples of head amine determinations, or for one or two independent samples for ^{3}H emissions. To compare head contents of β-alanine and histamine between wild-type and mutant flies, we used ANOVA followed by a Tukey's HSD test, by means of Systat 5.2.1 software (Systat, Chicago, IL, USA).

To analyse ERG recordings, the 'on' transient, 'off' transient and sustained negative response (SNR) were averaged for three or four of the ERG series obtained per fly, thus providing a mean of means for all variables. We then normalized the mean 'on' and 'off' transients relative to the SNR for each fly. To eliminate outliers, all normalized transients were averaged for each condition, and flies with transients of >2 s.d. were removed. For each condition, recordings from 12–23 flies having both normalized transients within the standard range were then pooled for statistical analysis. To compare transients for wild-type (*OR*), $su(r)^1r^C$ and $su(r)^1r^C;b^1$ flies under the two treatment conditions (those fed a solution of either 4% glucose or 5% β-alanine in 4% glucose), we used ANOVA followed by Student's *t*-test.

Immunohistochemistry and confocal microscopy

For immunohistochemistry, heads were fixed in 4% formaldehyde (as paraformaldehyde), embedded in OCT (Sakura Finetechnical, Tokyo, Japan) and 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). Sections were processed for single or double immunolabelling using the following primary antibodies: β-alanine (at 1:1000), β-alanyl-histamine (carcinine, at 1:100), splice variant B of *Drosophila* DVMAT-B (1:50) and β-galactosidase (β-gal, at 1:50). Polyclonal antibody (Abcam, catalogue no. ab37076-50, lot 700794; Cambridge, MA, USA) was raised in rabbit against β-alanine conjugated to BSA with glutaraldehyde; its specificity on *Drosophila* tissue was confirmed by the absence of immunolabelling after preadsorption with β-alanine, and by changes in signal after treatments reported in Results. A rat polyclonal antibody was raised against a concatemer of β-alanyl-histamine conjugated to keyhole limpet haemocyanin (KLH). Its specificity in *Drosophila* was confirmed by us from the lack of signal after preadsorption with carcinine, but not after histamine or β-alanine, all at 10^{-3} to 10^{-5} mol l^{-1}; immunolabelling was reduced in the mutant $ebony^1$ (data not shown). To generate antibody against VMAT-B, a peptide representing the last 21 amino acids of DVMAT-B (SVPDSDAEAGRTNEAYESERL, B1-peptide) was synthesized, HPLC purified, conjugated to KLH and then injected into rats (Romero-Calderón et al., 2008). Its specificity has been confirmed by reduced immunolabelling in the *Δ14* excision allele of *dVMAT* (Romero-Calderón et al., 2008). Mouse monoclonal antibody 40-1a (Developmental Studies Hybridoma Bank, Iowa City, IA, USA) was raised against β-gal and labels *lacZ*-expressing *Drosophila* cells, but not brains lacking *UAS-lacZ*.

The following secondary antibodies were used: Cy-3-conjugated goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA, USA) at 1:400; Alexa Fluor 488 goat anti-mouse (Molecular Probes, Eugene, OR, USA) at 1:100; and Alexa Fluor 488 goat anti-rat (Molecular Probes) at 1:100. Labelled sections were mounted in Vectashield and images collected with Zeiss LSM 410 or 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, labelled with β-alanine, were collected using the same confocal operating parameters for brightness and contrast throughout.

RESULTS

To resolve how β-alanine is recycled in the fly's visual system, we first made determinations of this β-amino acid and its histamine conjugate, carcinine, in wild-type flies and in different mutants for the enzymes involved in β-alanine biosynthesis.

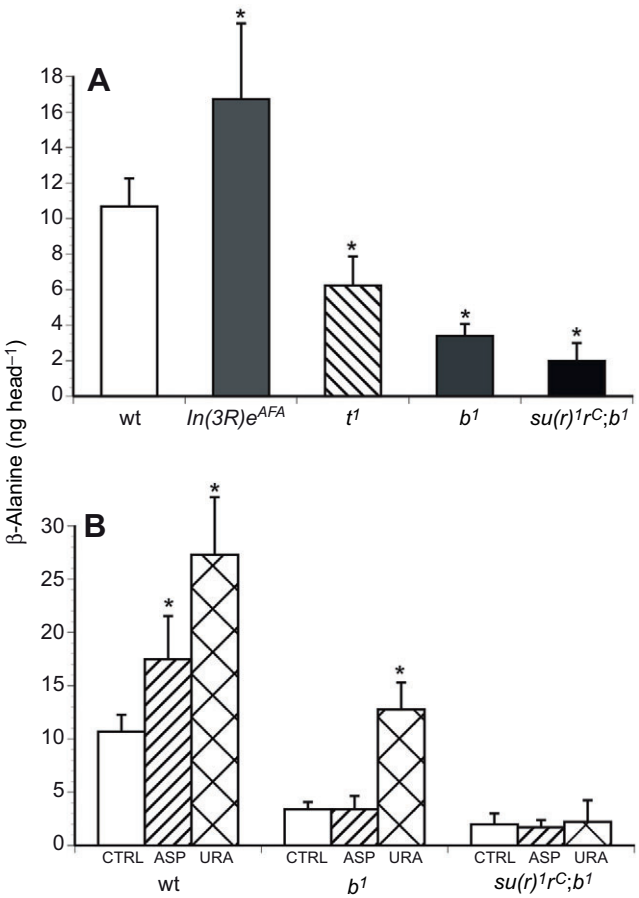

Fig. 2. (A) Head β-alanine (β-ala) in different mutants, relative to wild-type. Mutant *ebony* [as the homozygous inversion $In(3R)e^{AFA}$ (Caizzi et al., 1987)] has significantly more β-alanine than wild-type (wt), while tan (t^1) has significantly less, as with increasing severity do *black* (as b^1) and the Pyd1 Black double mutant [as $su(r)^1r^C;b^1$]. Differences from wild-type are significant (*P<0.05). (B) Total head β-alanine content in wild-type, and mutant *black* (as b^1) and double mutant *pyd1*; *black* [as $su(r)^1r^C;b^1$]. Values are from control flies (CTRL) and, relative to these, from flies fed different β-alanine substrates, either 5% L-aspartate (ASP) or 5% uracil (URA). These caused head β-alanine content to increase (*P<0.05) in wild-type and *black* mutant flies, but not in the double mutant.

Head β-alanine content

The β-alanine content (Fig. 2A) in the head of the wild-type fly averaged 11 ng, roughly five times the previously reported amount of histamine in the fly head (Borycz et al., 2002). The mutant *ebony* [as $In(3R)e^{AFA}$ (Caizzi et al., 1987)], which cannot conjugate β-alanine to histamine, had significantly more β-alanine than this, up to 17 ng $head^{-1}$; tan^1, which cannot hydrolyse β-alanyl-histamine to yield free histamine and β-alanine, had significantly less. The mutant *black* (b^1) had 68% less head β-alanine content than the wild-type, while the triple mutant $su(r)^1r^C;b^1$ had 81% less. Thus, head β-alanine was diminished by mutant genes of both *black* and *pyd1* acting in an additive fashion, while *ebony* and *tan* had reciprocal effects, as for many of their other actions on components of the histamine recycling pathway (Stuart et al., 2007).

Analysis of the pathways for β-alanine biosynthesis

To examine the action of the two synthetic pathways, *via* Black and Pyd1, respectively, we next allowed flies 24 h to drink one of the two respective substrates for these enzymes, either 5% aspartic acid

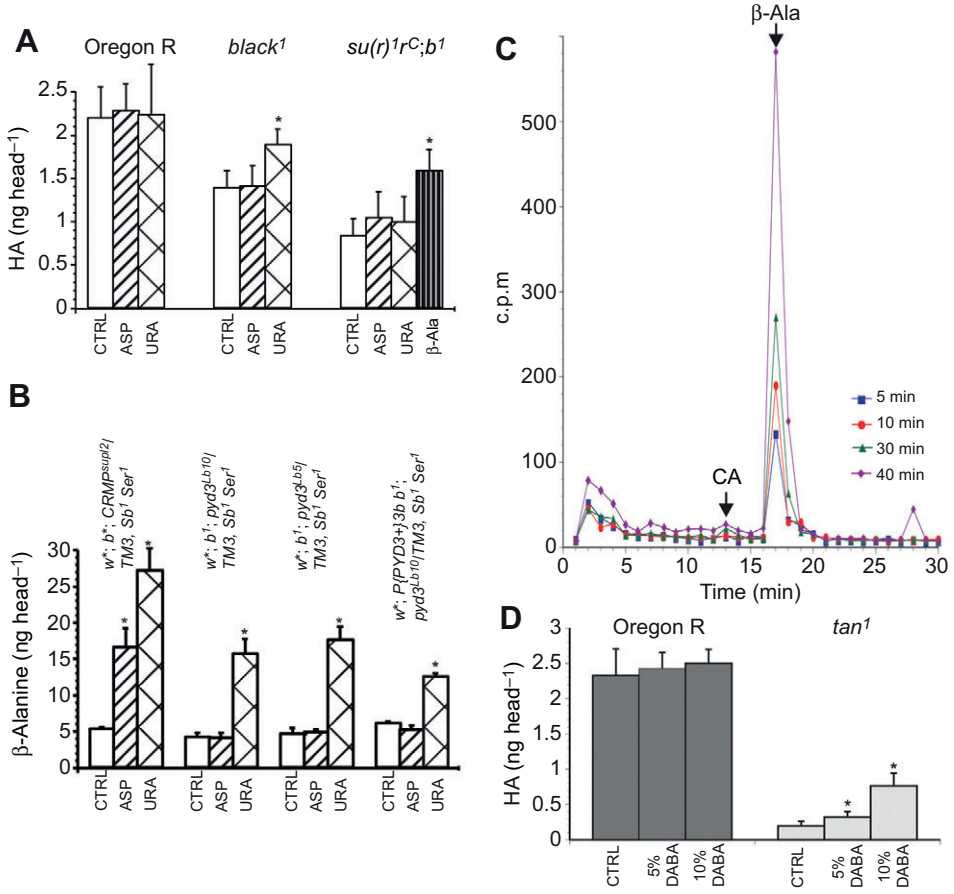

Fig. 3. (A) Total head histamine (HA) in wild-type (Oregon R), mutant *black* (as *black*^{1}) and double mutant *pyd1*; *black* [as *su(r)*^{1}*r*^{C}; *b*^{1}]. Values are from control flies and, relative to these, from flies fed either β-alanine (β-Ala) or one of two different substrates, L-aspartate (ASP) or uracil URA). In general, head histamine did not differ from the corresponding control (CTRL) values after feeding for any genotype, except for the content in mutant *black*^{1} measured after drinking uracil and double mutant *su(r)*^{1}*r*^{C}; *b*^{1} after drinking β-alanine, in which there was a significant increase (*P<0.05). (B) Head β-alanine in four double mutant genotypes for *black* and either *pyd2* or *pyd3*, either controls (CTRL) or flies given solutions of one of two different substrates, L-aspartate (ASP) or uracil URA), for 2 h. Uracil caused a significant increase in head β-alanine in all genotypes (*P<0.05) while aspartate caused an increase in head β-alanine in the *pyd2* single mutant (first genotype), but this was smaller than the increase with uracil. (C) Distribution of ^{3}H in the heads of *tan*^{1} flies after drinking 25% [^{3}H]β-alanine for 5–45 min, measured as radioactive scintillations (counts min^{-1}, CPM) in individual 1 min HPLC fractions. A clear peak corresponding to the retention time of β-alanine (β-ala) increases with the time during which flies could drink from the radiolabelled solution. A small peak of ^{3}H-labelled carcinine (CA) appears at a retention time of 13 min in the chromatogram. After 30 min of drinking ^{3}H-labelled β-alanine the carcinine peak was 22.6 CPM, 2.03 times more than the mean baseline rate for 10–16 min (less the 13 min peak) of 11.12 CPM. After 45 min drinking, the peak was 27.4 CPM, 1.34 times higher than the corresponding baseline level. These peaks are to be compared with those in flies that drank [^{3}H]histamine, as previously reported (Borycz et al., 2002). (D) Total head content of histamine (HA) in wild-type and *tan*^{1} flies fed diaminobutyric acid (DABA) at two different concentrations relative to controls (CTRL). No changes were seen in wild-type flies (Oregon R), but in *tan* both concentrations of DABA produced significant increases in head histamine (*P<0.05).

or 5% uracil, suspended in a 4% solution of glucose. Wild-type flies given aspartic acid showed increased head β-alanine, with 63% more than flies given 4% glucose alone (Fig. 2B). Wild-type flies that drank uracil showed an even greater increase in their head β-alanine, increasing by 155% over control flies. This difference indicates that uracil drives the fly's synthesis of β-alanine about twice as effectively as does aspartate.

This conclusion concurs with that of Hodgetts (Hodgetts, 1972) using radiolabelled precursors for β-alanine synthesis, but the result is nevertheless surprising because synthesis of β-alanine from aspartate is a simple one-step reaction, unlike the more complex three-step synthesis from uracil. Moreover, a high-affinity neuronal transporter of aspartate has been identified in *Drosophila* (Besson et al., 2000) and Black is co-expressed in the epithelial glia along with Ebony (Phillips et al., 1993; Richardt et al., 2002); both pieces of evidence support a leading role for aspartate in β-alanine synthesis in the eye, but do not address other compartments in the head. Mutant *black* (Fig. 2B) flies were unable to synthesize additional β-alanine when given aspartic acid as above, whereas 5% uracil strongly increased their head β-alanine content, by 276% relative to controls. An increase was visible even after 3 or 6 h of drinking uracil (data not shown). Mutant *su(r)*^{1}*r*^{C}; *b*^{1} flies, in contrast, displayed no increase in head β-alanine, either after drinking aspartic acid or when given uracil, indicating that the two pathways regulated by these genes both contribute to β-alanine biosynthesis (Fig. 2B). This failure on the part of *su(r)*^{1}*r*^{C}; *b*^{1} to produce excess β-alanine in the presence of exogenous uracil or aspartate indicates that some of the head β-alanine remaining in all *su(r)*^{1}*r*^{C}; *b*^{1} determinations, both controls and those from flies fed one of the two substrates, may be the product of (an)other enzyme(s), possibly derived from intestinal bacteria, or inadvertently supplied with the fly's food.

Reductions in head β-alanine in the mutants were paralleled by similar reductions in head histamine (Fig. 3A), as expected given that histamine recycling must depend critically on β-alanine availability.

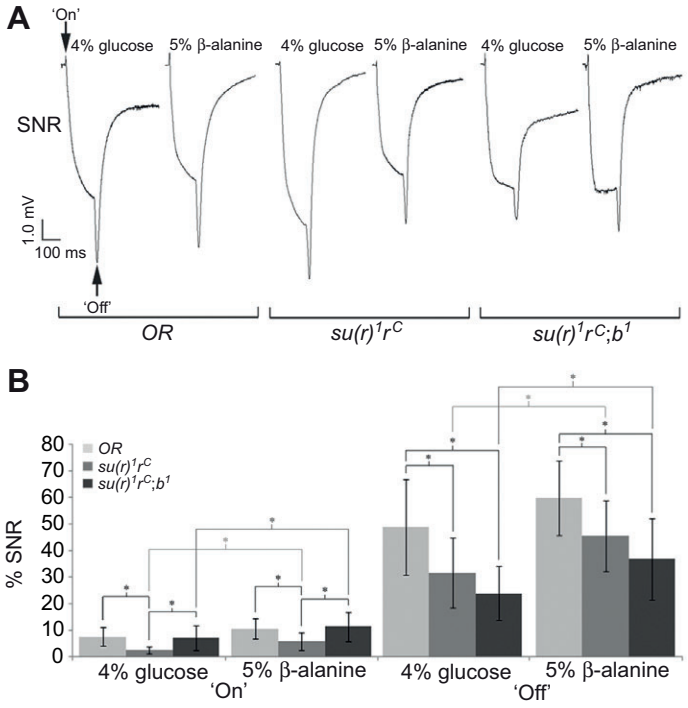

Fig. 4. β-Alanine concentration affects the size of the 'off' transient of the electroretinogram (ERG). ERGs were recorded for wild-type *OR*, a double mutant of the uracil-based pathway of β-alanine production $su(r)^1r^C$ and the triple mutant $su(r)^1r^C;b^1$, which affects both β-alanine production pathways. (A) Representative ERGs averaged from 10 light flashes at 20 s intervals for *OR*, $su(r)^1r^C$ and $su(r)^1r^C;b^1$ females after they drank a solution of 4% glucose or 5% β-alanine in 4% glucose. The 'on' and 'off' transients, as well as the sustained negative response (SNR), are indicated on the trace for glucose-fed *OR* flies. (B) Mean normalized 'on' and 'off' transients for *OR*, $su(r)^1r^C$ and $su(r)^1r^C;b^1$ flies. Error bars: ±1 s.d. Differences are significant (*P<0.05) within (black) and between (grey) treatments for both 'on' and 'off' transients.

Mutant *black* flies had a 36% increase in their head histamine when given 5% uracil, which indicates that the histamine deficit in this mutant can be partially rescued by means of uracil metabolism. Predictably, *black* flies were unable to use aspartate as a substrate, and showed no difference from controls in their head histamine content when fed this substrate (Fig. 3A). In the triple mutant $su(r)^1r^C;b^1$, the head histamine content in control flies was significantly less than in wild-type, and like in the single-mutant *black* it was not significantly altered by drinking either aspartate or uracil (Fig. 3A). In contrast, when the flies drank a solution of β-alanine, their head histamine did show a significant increase (P<0.05), albeit not to the extent observed for wild-type heads (Fig. 3A).

To conclude the mutant approaches to β-alanine biosynthesis, we also determined head β-alanine in double mutants of *black* and *pyd2* (CRMP) or *pyd3*, a β-alanine synthase gene that affects the final step in β-alanine synthesis from uracil (Fig. 1, Fig. 3B). These mutants were: (1) $w^*;b^*;CRMP^{supl2}/TM3$, Sb^1 Ser^1; (2) $w^*;b^1;pyd3^{Lb10}/TM3$, Sb^1 Ser^1; (3) $w^*;b^1;pyd3^{Lb5}/TM3$, Sb^1 Ser^1; and (4) $w^*;P\{PYD3+\}3b$ $b^1;pyd3^{Lb10}/TM3$, Sb^1 Ser^1. Mutant $w^*;b^*;CRMP^{supl2}/TM3$, Sb^1 Ser^1 displayed at least a 60% increase in head β-alanine when given a 5% solution of aspartic acid, suggesting that *b** is not a null mutation, and an increase of ~320% when given a 5% solution of uracil. The other mutants were also still able to synthesize β-alanine when given uracil to drink but not when they were given L-aspartic acid. Thus, relative to controls, $pyd3^{Lb10}/TM3$, Sb^1 Ser^1 had an increase in head β-alanine of 270% when given uracil, while $w^*;b^1;pyd3^{Lb5}/TM3$, Sb^1 *Ser1* showed an increase of 300%. Surprisingly, after *pyd3* rescue in $w^*;P\{PYD3+\}3b$ $b^1;pyd3^{Lb10}/TM3$, Sb^1 Ser^1 there was an increase of only 112% after uracil (Fig. 3B), less than in wild-type flies, which showed an increase of 155% in their head β-alanine when fed uracil *vs* glucose (Fig. 2B). The differences between the mutant and rescue values imply that the flies are not true nulls for either *pyd2* or *pyd3*, while simultaneously confirming that $black^1$ is, by contrast, a true null for aspartate decarboxylase. This conclusion rests on there being of a linear pathway for β-alanine biosynthesis and on the absence of an alternative to Pyd3 function.

β-Alanine uptake and the fate of [^{3}H]β-alanine in *tan*

We have previously shown that when tan^1 flies drink a tritiated histamine solution they accumulate tritiated carcinine in the head to a level at three times that of background, because they are unable to hydrolyse this metabolite to liberate histamine and β-alanine (Borycz et al., 2002). In a reciprocal extension of this finding, we gave tan^1 flies a droplet of 25% [^{3}H]β-alanine (37 MBq ml^{-1} and 1850 GBq mmol l^{-1}) and found they were also able to accumulate [^{3}H]carcinine within a period of 5–45 min (Fig. 3C). Thus, the increase in radioactivity at the retention time corresponding to carcinine peaked to twice that of the background, indicating that some of the radioactive β-alanine had been conjugated to histamine and thus recycled *via* the Ebony–Tan pathway.

Knowing that GABA and β-alanine have a very similar chemical structure, and that uptake or transport mechanisms for β-alanine might therefore share similarities with those for GABA in *Drosophila*, flies were also administered a known GABA transporter inhibitor DABA to see whether this might inhibit β-alanine uptake as well, and so in turn reduce the rate of histamine recycling (Fig. 3D). DABA was administered in two concentrations (5% and 10%, in 4% aqueous glucose) and these exhibited graded effects, but did not increase head histamine in the wild-type significantly. By contrast, the same doses of DABA generated significant increases in head histamine in *tan*. This mutant normally has only about one-tenth the content of head histamine of the wild-type fly (control value in Fig. 3D) (see also Borycz et al., 2002). This reduction occurs because *tan* flies are unable to hydrolyse carcinine, so that most head histamine remains locked in the reserves of this metabolite. Thus, DABA acted to increase this residual head histamine, and drinking 10% DABA gave an approximately fourfold increase in head histamine compared with head histamine in control *tan* flies that did not drink DABA.

β-Alanine affects the size of the ERG 'on' and off' transients

Mutant $su(r)^1r^C;b^1$ flies are unable to produce β-alanine because they lack both aspartate-dependent ($black^1$) and uracil-dependent ($su(r)^1r^C$) biosynthesis pathways. They nevertheless still had ERG 'on' and 'off' transients (Fig. 4A), proposed to derive primarily from the response of the lamina monopolar neurons to light (Heisenberg, 1971; Coombe, 1986). However, the 'off' transients were reduced (P<0.05) under control conditions in which $su(r)^1r^C$ or $su(r)^1r^C;b^1$ flies drank 4% glucose. The size of the normalized 'off' transient was reduced to 65% of *OR* wild-type in the double mutant $su(r)^1r^C$, and further in the triple mutant, $su(r)^1r^C;b^1$, to 49% of *OR*. While $su(r)^1r^C;b^1$ flies fed either glucose or β-alanine displayed no difference in their 'on' transients relative to *OR* (Fig. 4B), $su(r)^1r^C$ flies mutant for uracil-dependent production of β-alanine had reduced 'on' transients relative to both *OR* and $su(r)^1r^C;b^1$ (P<0.05).

When all of *OR*, $su(r)^1r^C$ and $su(r)^1r^C;b^1$ consumed 5% β-alanine in 4% glucose, the ratios of both the normalized 'on' and 'off'

transients increased compared with those of the glucose-fed flies of the same genotype. Wild-type 'off' transients increased to 122% after consuming β-alanine, albeit less than mutants. Significant changes were observed only in the transients of β-alanine-fed $su(r)^1r^C$ ('on': 239%; 'off': 144%) and $su(r)^1r^C;b^1$ ('on': 160%; 'off': 154%) flies relative to their glucose-fed counterparts. In fact, after consuming 5% β-alanine, $su(r)^1r^C;b^1$ flies were able to generate 'off' transients of similar size to those of glucose-fed *OR* flies ($P>0.05$).

Distribution of head β-alanine

The previous experiments were able to quantify the whole-head content of β-alanine and histamine after metabolic precursors of β-alanine had been administered to flies, but they failed to reveal the actual distribution of β-alanine and its conjugate carcinine within the visual system. For this, we used immunocytochemistry to reveal the cells in the visual system that contain β-alanine and carcinine, and thereby the pathways through which recycling could occur.

Immunolabelling for β-alanine in the wild-type fly (Fig. 5A) showed a diffuse signal throughout the visual system with the highest density in two layers of the retina, and in the photoreceptors and their terminals, at least those of R1–R6 in the lamina. In the compound eye, an unusual pattern of immunolabelling occurred just beneath the corneal lenses, in a layer corresponding to the two primary pigment cells, the four cone cells and the pseudocones these encircle (Ready et al., 1976). The very high signal in this layer and the specificity of the antibody that detected it suggested that these cells could serve as a storage site for the β-amino acid. A second layer of signal at the level of the basement membrane was partly attributable to the autofluorescence seen at this level in all preparations, but had an additional component that appeared to arise from the end feet of cells from the distal ommatidium that enlarge above the basement membrane, or from the fenestrated glia that lie beneath these (Fig. 6C′).

To confirm the cellular expression pattern of β-alanine first required us to confirm the specificity of its immunolabelling, as opposed to that of any of its closely related metabolites. A number of pieces of evidence provide assurance on this point. First, the signal for β-alanine immunolabelling was increased in wild-type flies 2 h after they drank 0.5% histamine (Fig. 5B). This increase indicated that β-alanine could be synthesized, as we also demonstrated by HPLC measurements of head content (data not shown), and its concentration in a proposed storage site increased, on demand, depending on the availability of a biogenic amine substrate. Second, flies mutant for *black*, which lack aspartate decarboxylase (Fig. 5E) and therefore one of the pathways for β-alanine biosynthesis, as well as for $su(r)^1r^C;b^1$ (*pyd1*; $black^1$) (Fig. 5F), which lacks both pathways, both had strongly reduced β-alanine signal in the head. The reduction in signal provides good evidence for the specificity of the primary antibody. The signal was not completely extinguished, however, any more than β-alanine was completely depleted in the head. Such depletion is almost impossible to procure because of the multiple pathways for β-alanine biosynthesis (Hodgetts, 1972). Third, the mutants $In(3R)e^{AFA}$ and tan^1 (Fig. 5C,D), which are unable to utilize β-alanine for carcinine synthesis (*ebony*), or to liberate β-alanine from stored carcinine (*tan*), had reciprocal effects on head β-alanine, with $In(3R)e^{AFA}$ increasing and tan^1 decreasing this β-amino acid. Fourth, in addition to pre-adsorption with β-alanine, we also conducted pre-adsorption controls for both carcinine and L-alanine. In each case, we used three concentrations of synthetic antigen (10^{-3}, 10^{-4} and 10^{-5} mmol l^{-1}). The immunosignal was extinguished by pre-adsorption with β-alanine at all three concentrations (data not shown). This result did not confirm that the antibody failed to detect the related compounds carcinine and alanine, however. It was important to test the specificity of pre-adsorption to these because of the chemical relationship between β-alanine and both its histamine conjugate, carcinine, and its related amino acid, L-alanine. The antibody continued to give a detectable signal when it had been pre-adsorbed with either of these, however, indicating that no part of the immunosignal arose by binding to either. These tests were of particular importance given that carcinine is known to form in the lamina's epithelial glia and L-alanine is expected to be a normal component of retinal pigment cells (Tsacopoulos et al., 1994).

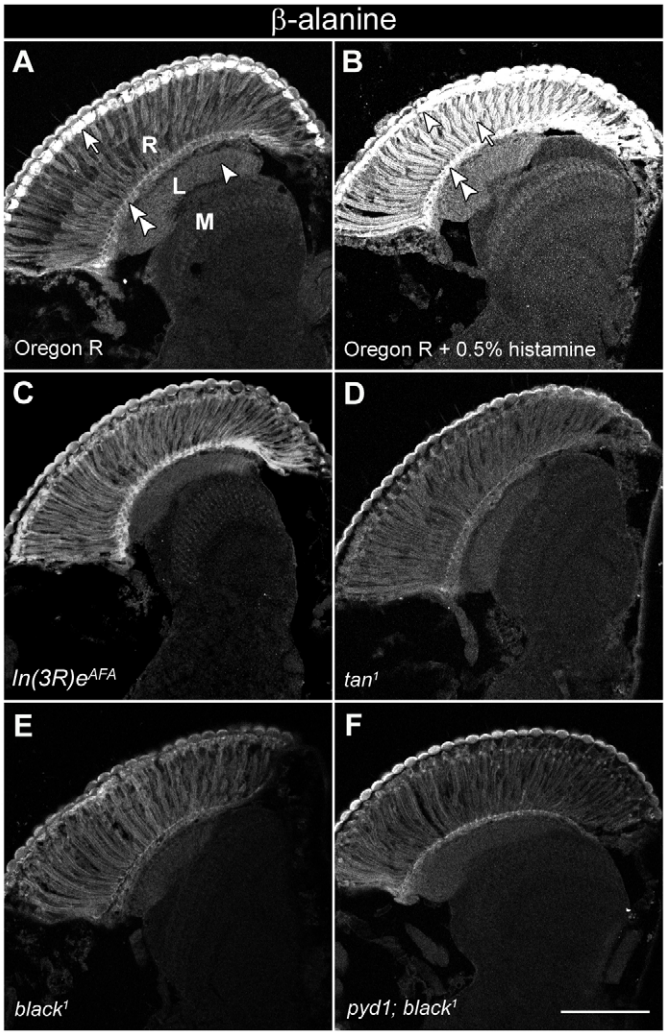

Fig. 5. Distribution of immunoreactivity to β-alanine in the heads of wild-type and mutant *Drosophila*. (A) Wild-type Oregon R shows label in the photoreceptors, especially in the lamina (arrowhead), a layer beneath the cornea (arrow) and also along the basement membrane region (double arrowhead). (B) Wild-type Oregon R after drinking 0.5% histamine shows increased label in the photoreceptor cell bodies (arrow), especially in the sub-corneal (arrowhead) and basement membrane (double arrowhead) layers. (C) Mutant $In(3R)e^{AFA}$ shows a weaker signal in the subcorneal layer and a somewhat stronger signal in the retina compared with wild-type (in A). (D) Mutant tan^1 shows a pattern like that of $In(3R)e^{AFA}$ (in C), but with a weaker band of label at the level of the basement membrane. (E) Mutant $black^1$ and (F) double mutant *pyd1*; $black^1$ show no clear differences from each other or from $ebony^1$ [$In(3R)e^{AFA}$] and tan^1. R, retina; L, lamina; M, medulla. Scale bar, 100 μm for all panels.

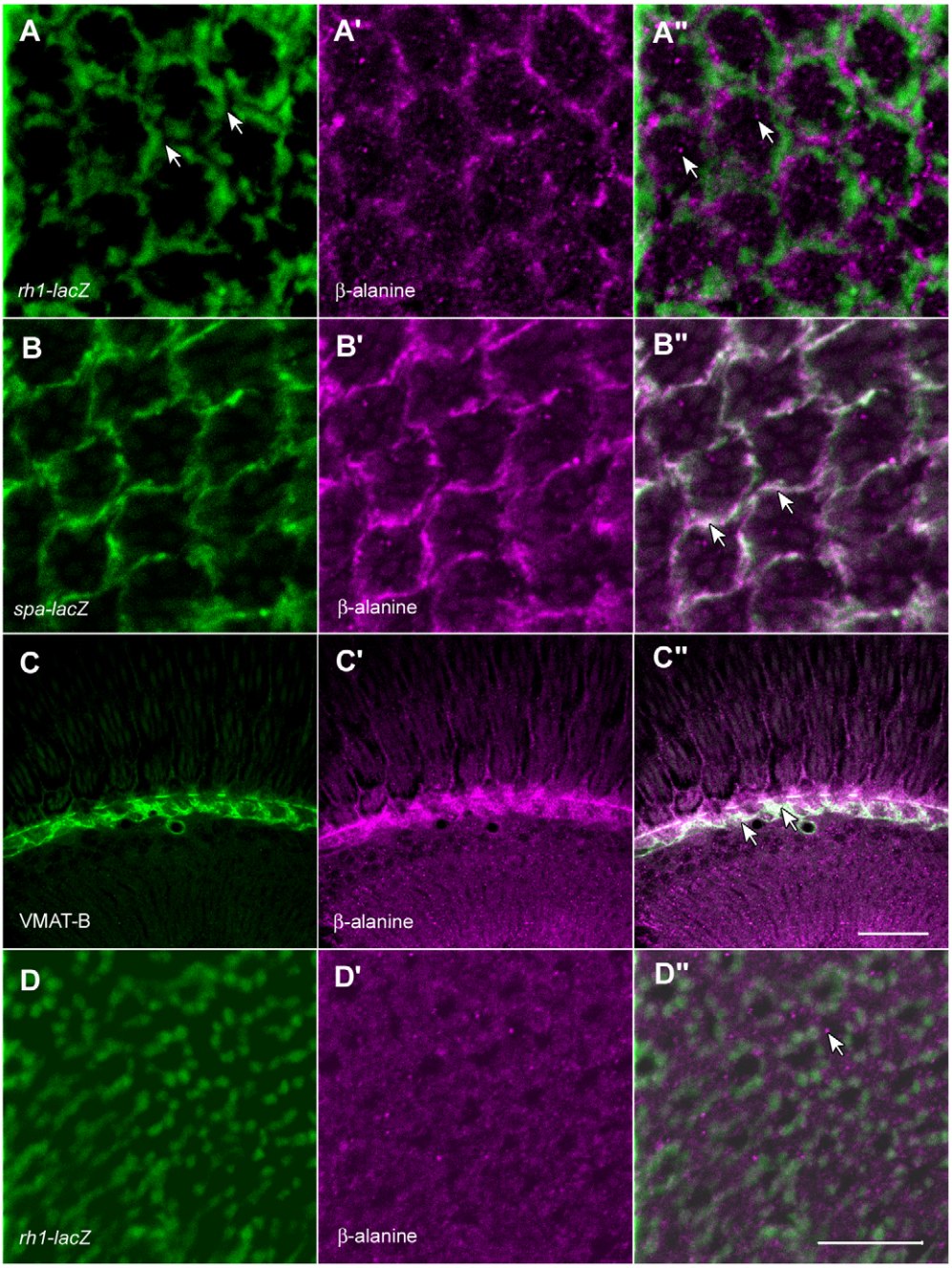

Fig. 6. (A) Immunoreactivity to β-alanine (magenta) and its distribution in identified cells and organelles of the wild-type *Drosophila* retina and lamina. (A) Photoreceptor cells in the retina labelled by expression of β-galactoside driven by *rh1-lacZ* (green). Compared with β-alanine, there is faint expression in photoreceptors (arrows in A″) but colocalization is mostly lacking, with β-alanine filling the gaps (arrows) between *rh1*-driven β-gal expression in the photoreceptors. (B) Pigment and cone cells in the retina, labelled by expression of β-galactoside driven by *spa-lacZ* (green), which overlaps extensively with β-alanine immunosignal in pigment cells (B′). (B″) Regions of overlap are extensive (arrows). (C) Immunoreactivity to VMAT-B (green) labels the fenestrated glia beneath the basement membrane, which overlaps with immunoreactivity to β-alanine (C′). (C″) Signal overlaps (arrows). Overlap is less clear in a region of β-alanine expression above the basement membrane that corresponds to the location of the pigment cell end feet. (D) Photoreceptor terminals R1–R6 in profiles of cross-sectioned lamina cartridges revealed by expression of β-galactoside driven by *rh1-lacZ* (green). Immunolabelling for β-alanine reveals signal of about equal strength in both the terminals of R1–R6 and the surrounding epithelial glial cells (D′). (D″) The brightest β-alanine puncta (arrow) lie mostly at the borders of R1–R6. Scale bar in D″, 10 μm for all A, B and D panels; scale bar in C″, 20 μm for all C panels.

Having confirmed the specificity of the β-alanine antibody, we attempted to localize the signal to specific cells within the visual system. Double labelling using anti-β-alanine with a marker for the different cell types was used to confirm the cellular origins of the label in the retina (Fig. 6): (1) *rh1-lacZ* labelled the outer photoreceptors R1–R6 (Mismer and Rubin, 1987); (2) *spa-lacZ*, a construct of *sparkling* (*spa*) was expressed in cone, primary pigment and bristle cells of pupal eye discs (Fu and Noll, 1997), and in young adults (U. Banerjee, unpublished); and (3) immunolabelling with VMAT-B (Romero-Calderón et al., 2008) detected the fenestrated glia beneath the basement membrane.

In the retina, although photoreceptors showed a faint immunosignal for β-alanine this signal did not overlap *rh1*-driven β-gal expression in the photoreceptors but instead labelled gaps between the photoreceptors, most conclusively seen in cross-sections of ommatidia (Fig. 6A–A″). These gaps appeared to be those occupied by pigment cells. Confirming this suggestion, β-alanine immunoexpression overlapped with *spa-lacZ*-driven β-gal expression (Fig. 6B–B″), indicating that β-alanine occurred at a relatively high concentration in pigment cells. The expression of β-alanine in cone cells (Fig. 5A) also overlapped with β-gal expression in longitudinal sections of *spa-lacZ* eyes (data not shown); and also colocalized with VMAT-B to fenestrated glia immediately beneath the basement membrane (Fig. 6C–C″). Thus, β-alanine extended through the depth of the retina and beneath the basement membrane to the first of the lamina's six layers of glia (Edwards and Meinertzhagen, 2010).

In addition to these specific sites of expression, β-alanine immunolabelled the photoreceptors and their terminals, both the terminals of R1–R6 (Fig. 6D–D″) in the lamina and those of R7

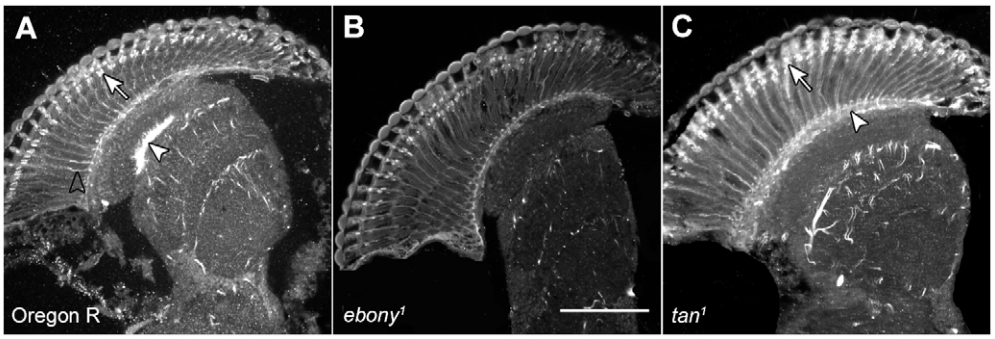

Fig. 7. Distribution of immunoreactivity to carcinine in the heads of wild-type and mutant *Drosophila*. (A) Wild-type Oregon R shows label in a layer beneath the cornea (arrow) and also in the marginal glia (filled arrowhead). The subcorneal layer probably includes primary pigment cells because slender immunolabelled profiles (open arrowhead) extend down to the basement membrane. Signal also appears in a subretinal layer. (B) Mutant *ebony*^{1} shows a reduction in signal in all parts of the brain, noticeable here in the optic lobe relative to the wild-type (in A). (C) Mutant *tan*^{1} shows label in the layers beneath the cornea (arrow) and basement membrane (arrowhead) that is stronger than in the wild-type; immunosignal in the subretinal layer probably arises from the fenestrated glia. Marginal glia are not labelled. Scale bar, 100 μm for all panels.

and R8 in the medulla, but seen clearly only after uptake of histamine (Fig. 5B). This label was not as bright at that in the two retinal layers identified above, but its photoreceptor location was not unexpected given the involvement of β-alanine in histamine recycling. In the lamina, there was additional signal between the cartridges, visible between the circles of R1–R6 terminals in cross-sections, i.e. expressed in the epithelial glia (Fig. 6D′). Expression in epithelial glia is consistent with the presence of β-alanine involved in the histamine shuttle pathway between R1–R6 terminals and epithelial glia, and was therefore also not unexpected, although the signal did not reveal these glia as exclusive storage sites for the β-amino acid.

Distribution of head carcinine

To identify the location of the β-alanine conjugate of histamine, we next undertook immunolabelling with a rat polyclonal antibody against carcinine. In the wild-type this labelled strongly in a layer at the proximal border of the lamina neuropile, among the marginal glia (Fig. 7A), in a subcorneal layer in the retina, which probably includes the primary pigment cells, as well as in a subretinal layer. Relative to these locations, *ebony*^{1} showed a marked reduction in all carcinine signals (Fig. 7B), compatible with its failure to accumulate carcinine, while *tan*^{1} showed an increased signal (Fig. 7C). That increase was obvious for the subcorneal layer, and also in the subretinal layer, which appeared to be fenestrated glia. In contrast, the marginal glia lacked signal (Fig. 7C).

β-Alanine colocalizes to carcinine in primary pigment cells

Given that both β-alanine and carcinine localize to sites beneath the cornea, we finally examined whether they were stored in the same cells of the ommatidium (Fig. 8). There was colocalization in primary pigment cells (Fig. 8B″), as well as in the photoreceptor neurons themselves (Fig. 8C″). In addition, β-alanine was found in two other sites – the secondary and tertiary pigment cells that surround the photoreceptors (Fig. 8C′) and in the pseudocone cavity between the primary pigment cells (Fig. 8B′), where it is possibly stored. Thus, both β-alanine and its conjugate carcinine are present in the retina, in cells that are not immediately involved in the local recycling of β-alanine from carcinine in the lamina. In addition to the photoreceptor cone, secondary and tertiary pigment cells all extend the depth of the retina from the cornea to the basement membrane.

DISCUSSION

Our findings provide evidence for the synthesis and recycling of β-alanine by multiple pathways in the fly's head, and that these pathways are important for the recycling of biogenic amines, especially histamine in the visual system. There, β-alanine assumes particular importance because photoreceptors contain large amounts of histamine that they release at high rates (Stuart et al., 2007). Histamine contributes a head content that is at least three times more than that of other biogenic amines, and most is of photoreceptor origin (Borycz et al., 2008). It is notable that the head content of β-alanine is, in turn, more than five times the content of histamine, so that abundant stores exist as potential sources of recyclable β-alanine. From the diffuseness of the brain's β-alanine immunosignal, much of this may be of non-photoreceptor origin, however. Its diffuse expression may simply reflect that, in addition to being a β-alanine precursor, uracil is also a nucleobase of RNA, and thus present in all living cells.

Our findings also provide new evidence that, in addition to the lateral shuttle pathway between photoreceptor and epithelial glial cells in the lamina, pathways for β-alanine and histamine recycling *via* carcinine must also exist in a vertical direction, between retina and lamina, and involve other lamina glia.

A retinal store of β-alanine in the *Drosophila* head

The pattern of immunolabelling for β-alanine in the visual system reveals an even distribution in the photoreceptors and optic lobe glia that in addition to any specific pathways for its synthesis is also compatible with other pathways involving β-alanine. For example, β-alanine is a product of the metabolism of uracil derived from RNA metabolism (Gojković et al., 2001). Unexpectedly, in this study we observed apparently high concentrations of β-alanine in the pigment cells and pseudocones of the *Drosophila* eye. These, we propose, are storage sites for this β-amino acid and therefore possibly important in amine recycling. Such storage would be a novel function for pigment cells, which have otherwise been thought of mainly as components of the light-screening apparatus of the ommatidium involved in light and dark adaptation (Autrum, 1981). Alternatively, β-alanine in the pigment cells may have a quite different function. For example, pigment cells in the honeybee ommatidium transform glucose to alanine, which they then shuttle to neighbouring photoreceptor cells to provide the metabolic substrate for phototransduction (Tsacopoulos et al., 1994). As a

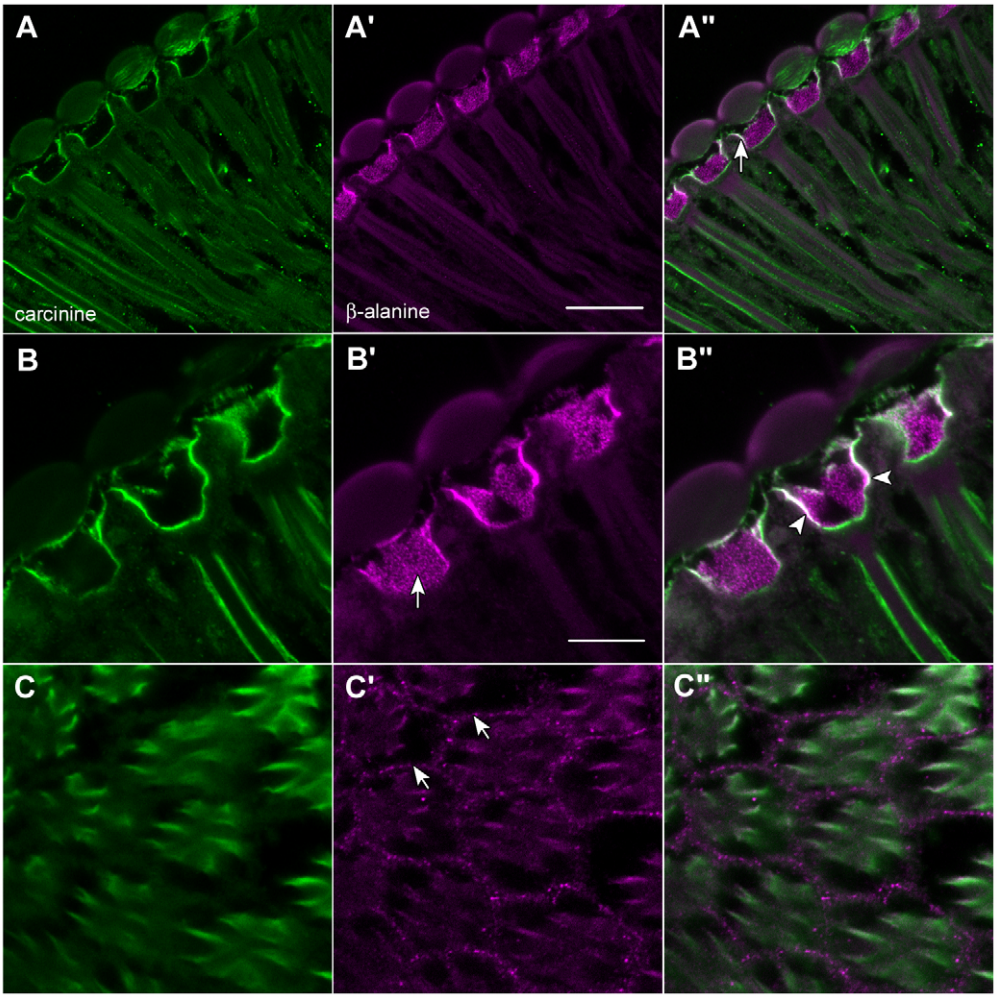

Fig. 8. Carcinine (green) and β-alanine (magenta) in the wild-type retina colocalize in primary pigment cells and photoreceptors. (A–A″) In longitudinal sections of the retina, the brightest signal for both epitopes is observed in the primary pigment cells (A″, arrow), shown enlarged in B–B″ (arrowheads in B″). In addition, β-alanine signal also occurs in the pseudocone cavities (arrow in B′). (C–C″) In cross-sections of ommatidia, additional sites of β-alanine signal are visible in the secondary and tertiary pigment cells that surround the ommatidium (arrows in C′), and colocalization with carcinine immunoreactivity is apparent in the photoreceptors (C″). Scale bar, 20 μm in A′ for all A panels; 10 μm in B′ for all B and C panels.

result, retinal pigment cells can be expected to store not only β-alanine but also its α-amino acid L-alanine, thus providing an interesting possible parallel to our findings. The specificity controls for our antibody exclude any crossover in immunodetection between these two sites, however.

Until now, histamine recycling *via* carcinine has been thought of as a lateral shuttle pathway between photoreceptor terminals and epithelial glia in the lamina (Stuart et al., 2007). Additional pathways have been postulated to involve the fenestrated glia (Romero-Calderón et al., 2008), which we now propose to be part of a longitudinal recycling pathway through the retina (Fig. 9). The discovery of a retinal storage site for β-alanine links three cell types in the retina (pigment, cone and photoreceptor cells) which all contain one or more of histamine, β-alanine and carcinine with the subretinal fenestrated glia, which contain both histamine and β-alanine but not carcinine. The presence of histamine and its two allied metabolites within the photoreceptors may be associated with cell body expression of Tan protein (Wagner et al., 2007; Aust et al., 2010), which alone can liberate histamine and β-alanine from carcinine, and thus provide a source for the β-alanine that accumulates in the photoreceptor. This explanation requires, however, that carcinine be able to reach the retina from the nearest sources of its synthesis by Ebony protein, the epithelial (Richardt et al., 2003) and proximal satellite glia, which lie near photoreceptor terminals (Edwards and Meinertzhagen, 2010). This separation between the site of carcinine synthesis and the site of its hydrolysis implies that carcinine must pass from the lamina to the retina, for which the simplest path is through the photoreceptor axoplasm (Fig. 9).

The longitudinal pathway *via* the retina poses the added problem of how carcinine might then pass from the photoreceptor cell body to the pigment cells to yield up its β-alanine, and for β-alanine to pass into the fenestrated glia, by way of the pigment cell end feet that abut them. Beneath the cornea, the two primary pigment cells abut six secondary and three tertiary pigment cells that in turn contact the photoreceptor neurons, but also have long extensions that contact these cells down the length of the ommatidium (Wolff and Ready, 1993). Although no obvious or direct connection exists to the glia in the lamina, the most obvious pathway would appear to be by way of the pigment cell end feet. Such a pathway needs to be identified, but apparently involves transport mechanisms other than diffusion through gap junctions, because dye injections into R1–R6 (S. R. Shaw, personal communication) label neither dye-coupled cone cells distally nor epithelial cells proximally. The possibility of specific transporters also exists.

Finally, *Drosophila* pigment cells are also necessary for chromophore regeneration (Wang et al., 2010). Together with our findings, it is clear that in addition to their role in screening stray light, these cells must serve important additional functions in vision.

Multiple synthetic pathways for head β-alanine

Previous studies have shown that flies have multiple pathways for the biosynthesis of β-alanine. Thus, Hijikuro first showed that

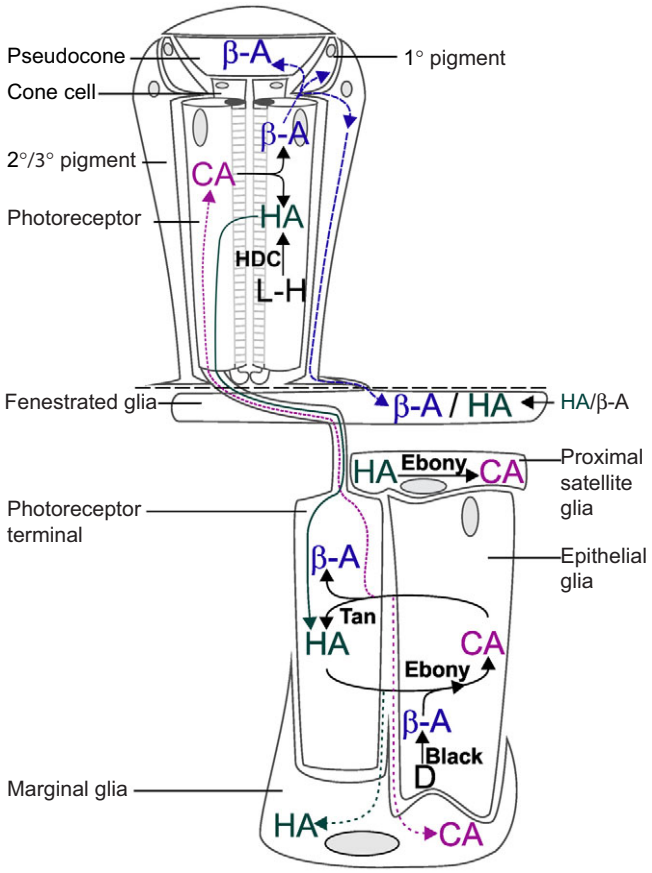

Fig. 9. Cellular localization of β-alanine (β-A, blue), carcinine (CA, magenta) and histamine (HA, green) in cells of the ommatidium and underlying lamina cartridge. At least some carcinine is proposed to diffuse in a retrograde direction back into the photoreceptor cell body (magenta arrow). β-Alanine is mostly found in the pseudocone and the pigment cells that surround it, and in fenestrated glia, where it may derive from the end feet of the pigment cells. Carcinine is clearly detectable in marginal glia, where it most likely derives from the neighbouring epithelial glia, which are Ebony expressing and therefore produce carcinine. β-Alanine occurs in a subretinal layer that probably contains the fenestrated glia and could provide a pathway for β-alanine to return to the lamina glia. Such a tentative pathway would depend on the presence and patency of gap junctions or of appropriate transporters both between the pigment and glial cells and among the glial cells. Alternatively, β-alanine may support some quite different function locally in the retina. Abbreviations: L-H, L-histidine; HDC, histidine decarboxylase; L-aspartate, D.

housefly pupae synthesize β-alanine from both radiolabelled L-aspartic acid and uracil (Hijikuro, 1968). Other precursors provide substrates for β-alanine biosynthesis, with housefly pupae utilizing not only uracil and aspartate but also pantothenate and propionate most efficiently for this purpose (Ross and Monroe, 1972). Strøman demonstrated that *su(r)* (*pyd1*) larvae were unable to synthesize β-alanine when fed on tritiated uracil whereas wild-type controls efficiently produce β-alanine from the same substrate (Strøman, 1974).

Our findings confirm the existence of these multiple pathways. Thus, mutant *black*^{1} that drink L-aspartic acid fail to show an increase in head β-alanine, which confirms that the product of this gene can indeed function as an aspartate decarboxylase and is consistent with *black*^{1} being a null mutation for that function. Moreover, although aspartate is in principle a precursor of uracil, it can be assumed that exogenous sources of L-aspartate given to flies are not used to synthesize uracil in amounts that would in turn induce increases in head β-alanine. Our results have shown that in the head both L-aspartate and uracil are substrates for the synthesis of β-alanine, with uracil being the more effective of these two as a source in *Drosophila*. Likewise, in the housefly Ross and Monroe report that 56% of tritiated uracil injected into pupae is incorporated into β-alanine while only 24% of tritiated aspartate is treated in this way (Ross and Monroe, 1972), so the greater capacity to utilize uracil over aspartate in β-alanine synthesis seems to be general in flies.

This flexibility may explain why *Drosophila black* mutants, despite the obvious cuticular melanization and significantly reduced aspartate decarboxylase activity, still have an ERG with normal transients and behavioural responses to visual cues (Phillips et al., 2005). However, the normal availability of uracil is apparently not sufficient to maintain head β-alanine at control levels as β-alanine is significantly reduced in *black*^{1}, but these levels must still be sufficient to supply the carcinine cycle and to support the normal ERG seen in this mutant. This sufficiency indicates that the role of *black* within the lamina's epithelial glia (Phillips et al., 1993) may be dispensable for vision (Phillips et al., 2005). We therefore infer that recycling is efficient and that even greatly diminished β-alanine levels are sufficient to maintain this recycling and to support synaptic transmission at photoreceptors, at least as revealed by the transients of the ERG in *black*^{1} (Phillips et al., 2005), as well as in *su(r)*^{1}*r*^{C};*b*^{1} flies, which have defects in one or both β-alanine biosynthesis pathways.

The relative roles of aspartate and uracil in the visual system

The fact that flies may use uracil to support β-alanine synthesis in the head does not contradict the normal role of aspartate for such synthesis in the visual system. Indeed the presence of Black in the epithelial glia (Phillips et al., 1993), co-expressed with Ebony (Richardt et al., 2002), provides good evidence for the hypothesis that the aspartate pathway is the major source of β-alanine in these cells. The normal ERG in *black* mutants (Phillips et al., 2005) indicates only that β-alanine derived from uracil is sufficient to maintain normal transmission at the first synapse, even though the increase in head histamine we see when *black*^{1} mutants drink 5% uracil only partially rescues the wild-type head histamine phenotype. However, we have previously shown that flies mutant for *black* show a large increase in head histamine when given β-alanine to drink, attaining a value seen in wild-type heads (Borycz et al., 2002). The difference between the effects on head histamine of administering uracil or its metabolic product β-alanine may suggest that the transport of uracil-derived β-alanine to the place where it is needed, i.e. the epithelial glia, is not sufficient to maintain normal histamine recycling. Despite this, our experiments in which flies drank uracil and L-aspartic acid demonstrate that they more effectively utilize uracil to synthesize β-alanine, even though under physiological conditions the supply of uracil can apparently be limited. Accurate measurement of the effects of supplying uracil is difficult, however, because of its low aqueous solubility. Moreover, supplying this substance orally frustrates precise calculations of dose–response functions, because uptake can only occur *via* the gut and haemolymph, which impose their own transfer kinetics. In addition, measurements of whole-head contents of β-alanine may be complicated by reactions that take place in the body. So, in this case Black protein, which is present in the cuticle, may readily have access to consumed L-aspartic acid from exogenous sources, and can then produce β-alanine, which is transported to the head *via* the haemolymph. However, Black protein in the epithelial glia is housed

beyond the restrictive blood–brain barrier, which may limit access to its substrate. In a similar vein, Pyd1 (as Dreg3) is concentrated exclusively in the head of adults (Van Gelder et al., 1995), possibly also within the glia (Altenhein et al., 2006), and so the blood–brain barrier's capacity to transport uracil may be a limiting factor in the production of β-alanine by the Pyd pathway.

Our previous finding (Borycz et al., 2002) that double mutant *tan*;*black* flies fail to concentrate radioactive carcinine when given a tritiated histamine solution to drink is compatible with the predominance of the aspartate over the uracil pathway for synthesis, at least within the *Drosophila* visual system. If this were not so, the double mutant would concentrate carcinine derived from the exogenous tritiated histamine and the β-alanine they synthesized from uracil. Rescue of the *tan* phenotype for carcinine accumulation in double mutant *tan*;*black* flies was, by contrast, easily obtained by supplying exogenous β-alanine (Borycz et al., 2002), indicating the differential distribution and/or transport of β-alanine – whether from uracil or not – in the fly's head, with the predominant use of the aspartate pathway in the visual system. A more accurate analysis of the role of the uracil pathway and its involvement, if any, in histamine recycling within the optic neuropiles requires precise localization of the proteins within the three-step Pyd-dependent pathway, which produces β-alanine from uracil.

Not only are there two main pathways for β-alanine synthesis but also these two interact. Thus, L-aspartate inhibits Pyd3, the final step in β-alanine synthesis from uracil (Andersen et al., 2008), and this interference may contribute to the smaller increase in head β-alanine seen after drinking 5% L-aspartic acid than that seen after drinking 5% uracil. Aspartate is also a substrate for the biosynthesis of uracil (Davidson and Kern 1994). Our mutant $su(r)^1r^C;b^1$ contained only 19% the wild-type amount of head β-alanine, much less than $black^1$ alone, which also indicates that a smaller percentage of this amino acid is required to maintain vital functions, and while administered β-alanine can rescue the $su(r)^1r^C;b^1$ histamine phenotype, it does so only to a limited extent. The difference in histamine content of fed $su(r)^1r^C;b^1$ when compared with the full rescue seen in single mutant *black* (Borycz et al., 2002) is difficult to explain. It may be because the *ebony* recycling pathway is down-regulated in the triple mutant to match its reduced histamine content, which relative to wild-type is even less than that in single mutant *black*. Alternatively, the congenital lack of β-alanine in the triple mutant relative to the single mutant may have long-term effects on neuronal and glial physiology that make it impossible to rescue the wild-type phenotype by simply supplying endogenous β-alanine later on.

Even in the triple mutant, β-alanine was never fully eliminated. Reduced β-alanine, as seen in our HPLC data, is possibly the reason that Pedersen described enhanced melanization in $su(r)^1r^C;b^1$ compared with b^1 (Pedersen, 1982). These show a further decrease in β-alanine in $su(r)^1r^C;b^1$ relative to b^1 and wild-type flies. In addition, these minimal concentrations of β-alanine are sufficient to maintain the ERG. Differences in the whole-head histamine and β-alanine content between OR and $su(r)^1r^C;b^1$ flies are reflected in the reduced size, but not elimination of, the ERG 'on' and 'off' transients in $su(r)^1r^C;b^1$ flies. 'Off' transients are reduced by eliminating the uracil-based pathway in $su(r)^1r^C$ and are further reduced by eliminating both β-alanine pathways in $su(r)^1r^C;b^1$, indicating that production of β-alanine modifies the visual response to light in a direct manner. A reduction of histamine to 40% of wild-type levels and of β-alanine to 19% of wild-type in $su(r)^1r^C;b^1$ corresponds to a decrease of the normalized 'off' transient to 49% of the wild-type. This decrease in the 'off' transient was rescued to almost wild-type levels by feeding $su(r)^1r^C;b^1$ flies a solution of β-alanine, suggesting that exogenous sources of β-alanine gain access to the visual system and alleviate mutant effects on the histamine recycling pathway that otherwise impair visual system physiology and function. Seemingly paradoxical, 'on' transients of $su(r)^1r^C$ flies but not $su(r)^1r^C;b^1$ flies are reduced relative to OR, suggesting that a mutation of the aspartate pathway, b^1, opposes mutations in the uracil pathway [$su(r)^1r^C$], at least for factors that control the 'on' transient. However, insofar as aspartate is a substrate for the biosynthesis of both β-alanine and uracil (Davidson and Kern, 1994), these pathways interact. Our finding that 'on' and 'off' transients are differentially affected endorses the separable origins of these transients as revealed by volatile anaesthetics (Rajaram and Nash, 2004).

β-Alanine transport and recycling

Our experiments in which flies drank ^{3}[H]β-alanine failed to confirm that this amino acid is recycled in a manner similar to that for histamine (Borycz et al., 2002), even though a small quantity is captured and detectable as radioactive carcinine. As previously reasoned (Stuart et al., 2007), the supply of histamine from its precursor L-histidine is probably very limited relative to the rate of histamine released, requiring the efficient recycling of histamine at the photoreceptor terminals. Unlike its treatment of histamine, *Drosophila* appears not to rely on recycling as a significant means to secure β-alanine but instead may have a sufficient supply *via* new synthesis from uracil or aspartate. The reason for this difference could simply be that β-alanine is so abundant, with more than 10 ng in the wild-type head, roughly five times more than that of histamine. The weakness of the β-alanine recycling we observed does not exclude the need to transport it between different brain compartments, however. Our findings show that the blockade of β-alanine transport by DABA strongly increases histamine in tan^1. This increase clearly suggests that some of this amino acid has to be transported to sites of carcinine production in the epithelial and proximal satellite glia, both sites of *ebony* expression (Richardt et al., 2003; Edwards and Meinertzhagen, 2010). With reduced β-alanine returning to these glia because of reduced transport, carcinine production would likewise be reduced, leaving more free histamine in an unconjugated state. Blockade of that transport is implicated because GABA and β-alanine share a similar chemical structure and in vertebrates GATs-2,3,4 transport both GABA and β-alanine (Tamura et al., 1995); β-alanine is, moreover, known to block the neuronal transport of GABA (Clark et al., 1992).

How is β-alanine returned to the lamina glia?

Our data identify new elements in the transfer of β-alanine between the eye and lamina. Although both the β-alanine and carcinine we find in the retina may be unrelated to the same compounds generated during histamine recycling in the lamina, the fact that glial and pigment cells harbour these compounds suggests that they may all be part of a vertical recycling pathway between the retina and lamina, as depicted in Fig. 9.

In principle, hydrolysis of carcinine by Tan could occur either in the terminal of the photoreceptor in the lamina – the site closest to carcinine return from the epithelial glia – or in the cell body itself, as both parts of the neuron express Tan (Wagner et al., 2007). Thus, β-alanine would diffuse retrogradely either before or after its hydrolysis from carcinine, although at least some carcinine must make its way to the soma to reveal immunosignal in that location. The larger volume of the cell body could then serve as a storage buffer. The size of that buffer may be 20 times larger than the corresponding lamina compartment [based on data in table 1 of Borycz et al. (Borycz et al., 2005)].

Tan's action, either in the lamina terminal or cell body of R1–R6, releases histamine for anterograde diffusion to, and vesicular repackaging in, the terminal. We propose that the β-alanine that is co-liberated with histamine in the photoreceptor cell body returns *via* a different path, *via* the pigment cells, their end feet and the fenestrated glia these contact. However, the pathways between pigment cells and fenestrated glia, and between fenestrated glia and proximal satellite glia, the first *en route* site of Ebony expression for the synthesis of carcinine, are not known. Such pathways may serve to return β-alanine directly to the epithelial glia, or may simply recycle β-alanine locally through the proximal satellite glia, which also express *ebony*; alternatively β-alanine may serve some additional function in the eye.

This scheme introduces several novel features. First, it invokes a vertical pathway for recycling histamine, β-alanine and their conjugate carcinine, between the lamina cartridge and its overlying ommatidium. This augments the predominantly lateral Ebony/Tan shuttle previously invoked in the lamina (Stuart et al., 2007). Although still highly speculative, the vertical pathway in turn introduces novel functions for its participating cells. These include an unexpected role for pigment cells, which not only prevent stray light from producing noise in the neighbouring photoreceptors (Autrum, 1981) but also support phototransduction *via* an alanine/glucose shuttle pathway reported in the honeybee ommatidium (Tsacopoulos et al., 1994), although yet to be confirmed in *Drosophila*. In addition, we now propose they also feed a pathway for the return of β-alanine to the lamina's epithelial glia.

In the proximal lamina, the marginal glia also store histamine and carcinine. Above them, *ebony*-expressing epithelial glia penetrate the terminals of R1–R6 to form specialized organelles, capitate projections, that are proposed integrated sites for recycling vesicle membrane and carcinine recovery (Fabian-Fine et al., 2003). Although they surround R1–R6 terminals (Edwards et al., 2011), marginal glia lack capitate projections, and Ebony expression, and are therefore probably not involved directly in carcinine and histamine recycling.

The histamine recycling pathway in photoreceptors faces two major physiological demands. First, histamine is released at high rates, which if unopposed would deplete the eye within seconds (Stuart et al., 2007). Second, the demands on histamine recycling vary greatly from moment to moment, at least within the time frame of 100 ms (Laughlin and Osorio, 1989), depending on the light stimulus conditions that result from the fly's own activity and changes in its ambient light conditions. To maintain a constant supply of histamine may therefore require not only a fast recycling pathway *via* carcinine but also storage sites for the neurotransmitter, as well as β-alanine and their conjugate carcinine. These storage sites seem to be the marginal and fenestrated glia and, in the retina, the pigment cells. The fenestrated glia have already been recognized as such a site, and three interrelated candidate functions, recycling, spillover and reserve, have been identified (Romero-Calderón et al., 2008). We imagine that a rapid reuptake pathway is processed *via* epithelial glia and their capitate projections in the lamina. The additional storage sites in the ommatidium and cartridge are possibly responsible for the slower supply of histamine to photoreceptors for re-release, the supply of β-alanine for synthesis of carcinine in the epithelial and proximal satellite glia, or the return of carcinine to the photoreceptor for the liberation of both.

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REFERENCES

Altenhein, B., Becker, A., Busold, C., Beckmann, B., Hoheisel, J. D. and Technau G. M. (2006). Expression profiling of glial genes during *Drosophila* embryogenesis. *Dev. Biol.* **296**, 545-560.

Andersen, B., Lundgren, S., Dobritzsch, D. and Piskur, J. (2008). A recruited protease is involved in catabolism of pyrimidines. *J. Mol. Biol.* **379**, 243-250.

Aust, S., Brüsselbach, F., Pütz, S. and Hovemann, B. T. (2010). Alternative tasks of *Drosophila* Tan in neurotransmitter recycling *versus* cuticle sclerotization disclosed by kinetic properties. *J. Biol. Chem.* **285,** 20740-20747.

Autrum, H. (1981). Light and dark adaptation in invertebrates. In *Handbook of Sensory Physiology*, Vol. VII/6C, *Comparative Physiology and Evolution of Vision in Invertebrates*, pp. 1-91. Berlin, Heidelberg: Springer-Verlag.

Besson, M.-T., Soustelle, L. and Birman, S. (2000). Selective high-affinity transport of aspartate by a *Drosophila* homologue of the excitatory amino-acid transporters. *Curr. Biol.* **10**, 207-210.

Blenau, W. and Baumann, A. (2005). Molecular characterization of the ebony gene from the American cockroach, *Periplaneta americana*. *Arch. Insect Biochem. Physiol.* **59**, 184-195.

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.

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., Borycz, J. A., Kubów, A., Lloyd, V. and Meinertzhagen, I. A. (2008). *Drosophila* ABC transporter mutants *white*, *brown* and *scarlet* have altered contents and distributions of biogenic amines in the brain. *J. Exp. Biol.* **211**, 3454-3466.

Borycz, J. A., Borycz, J., Kubów, A. Kostyleva, R. and Meinertzhagen, I. A. (2005). Histamine compartments of the *Drosophila* brain with an estimate of the quantum content at the photoreceptor synapse. *J. Neurophysiol.* **93**, 1611-1619.

Caizzi, R., Ritossa, F., Ryseck, R.-P., Richter, S. and Hovemann, B. (1987). Characterization of the *ebony* locus in *Drosophila melanogaster*. *Mol. Gen. Genet.* **206**, 66-70.

Clark, J. A., Deutch, A. Y., Gallipoli P. Z. and Amara S. G. (1992). Functional expression and CNS distribution of β-alanine-sensitive neuronal GABA transporter. *Neuron* **9**, 337-348.

Coombe, P. E. (1986). The large monopolar cells L1 and L2 are responsible for ERG transients in *Drosophila*. *J. Comp. Physiol. A* **159**, 655-665.

Curtin, K. D., Zhang, Z. and Wyman, R. J. (2002). Gap junction proteins are not interchangeable in development of neural function in the *Drosophila* visual system. *J. Cell Sci.* **115**, 3379-3388.

Davidson, J. N. and Kern, C. B. (1994). Revision in sequence of CAD aspartate transcarbamylase domain of *Drosophila*. *J. Mol. Biol.* **243**, 364-366.

Edwards, T. N. and Meinertzhagen, I. A. (2010). The functional organisation of glia in the adult brain of *Drosophila* and other insects. *Prog. Neurobiol.* **90**, 471-497.

Edwards, T. N., Nuschke, A. C., Nern, A. and Meinertzhagen, I. A. (2012). The organization and metamorphosis of glia in the *Drosophila* visual system. *J. Comp. Neurol.* (in press).

Evans, P. D. (1980). Biogenic amines in the insect nervous system. *Adv. Insect Physiol.* **15**, 317-473.

Fabian-Fine, R., Verstreken, P., Hiesinger, P. R., Horne, J. A., Kostyleva, R., Zhou, Y., Bellen, H. J. and Meinertzhagen, I. A. (2003). Endophilin promotes a late step in endocytosis at glial invaginations in *Drosophila* photoreceptor terminals. *J. Neurosci.* **23**, 10732-10744.

Fu, W. and Noll, M. (1997). The Pax2 homolog *sparkling* is required for development of cone and pigment cells in the *Drosophila* eye. *Genes Dev.* **11**, 2066-2078.

Gojković, Z., Sandrini, M. P. B. and Piškur, J. (2001). Eukaryotic β-alanine synthases are functionally related but have a high degree of structural diversity. *Genetics* **158**, 999-1011.

Hardie, R. C. (1987). Is histamine a neurotransmitter in insect photoreceptors? *J. Comp. Physiol. A* **161**, 201-213.

Heisenberg, M. (1971). Separation of receptor and lamina potentials in the electroretinogram of normal and mutant *Drosophila*. *J. Exp. Biol.* **55**, 85-100.

Hijikuro, S. (1968). Studies on the biosynthetic pathways of β-alanine in wild and black puparium strains of the housefly. *Jpn. J. Genet.* **43**, 419.

Hodgetts, R. B. (1972). Biochemical characterization of mutants affecting the metabolism of β-alanine in *Drosophila*. *J. Insect Physiol.* **18**, 937-947.

Jacobs, M. E. (1968). β-Alanine use by *ebony* and normal *Drosophila melanogaster* with notes on glucose, uracil, DOPA and dopamine. *Biochem. Genet.* **1**, 267-275.

Jacobs, M. E. (1980). Influence of β-alanine on ultrastructure, tanning, and melanization of *Drosophila melanogaster* cuticles. *Biochem. Genet.* **18**, 65-76.

Laughlin, **S. B. and Osorio**, **D.** (1989). Mechanisms for neural signal enhancement in the blowfly compound eye. *J. Exp. Biol.* **144**, 113-146.

Lundgren, S., Lohkamp, B., Andersen, B., Piskur, J. and Dobritzsch, D. (2008). The crystal structure of β-alanine synthase from *Drosophila melanogaster* reveals a homooctameric helical turn-like assembly. *J. Mol. Biol.* **377**, 1544-1559.

Matthews, M. M. and Traut, T. W. (1987). Regulation of N-carbamoyl-β-alanine amidohydrolase, the terminal enzyme in pyrimidine catabolism, by ligand-induced change in polymerization. *J. Biol. Chem.* **262,** 7232-7237.

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.
Mismer, D. and Rubin, G. M. (1987). Analysis of the promoter of the *ninaE* opsin gene in *Drosophila melanogaster*. *Genetics* **16**, 565-578.
O'Tousa, J. E., Baehr, W., Martin, R. L., Hirsh, J., Pak, W. L. and Applebury, M. L. (1985). The *Drosophila* ninaE gene encodes an opsin. *Cell* **40**, 839-850.
Pedersen, M. B. (1982). Characterization of an X-linked semi-dominant *suppressor of black Su(b)* (1-55.5) in *Drosophila melanogaster*. *Carlsberg Res. Commun.* **47**, 391-400.
Phillips, A. M., Salkoff, L. B. and Kelly, L. E. (1993). A neural gene from *Drosophila melanogaster* with homology to vertebrate and invertebrate glutamate decarboxylases. *J. Neurochem.* **61**, 1291-1301.
Phillips, A. M., Smart, R., Strauss, R., Brembs, B. and Kelly, L. E. (2005). The *Drosophila black* enigma: the molecular and behavioural characterization of the *black*[1] mutant allele. *Gene* **351**, 131-142.
Piskur, J., Schnackerz, K. D., Andersen, G. and Björnberg, O. (2007). Comparative genomics reveals novel biochemical pathways. *Trends Genet.* **23**, 369-372.
Rajaram, S. and Nash, H. A. (2004). A specific alteration in the electroretinogram of *Drosophila melanogaster* is induced by halothane and other volatile general anesthetics. *Anesth. Analg.* **98**, 1705-1711.
Rawls, J. M., Jr (2006). Analysis of pyrimidine catabolism in *Drosophila melanogaster* using epistatic interactions with mutations of pyrimidine biosynthesis and β-alanine metabolism. *Genetics* **172**, 1665-1674.
Ready, D. F., Hanson, T. E. and Benzer, S. (1976). Development of the *Drosophila* retina, a neurocrystalline lattice. *Dev. Biol.* **53**, 217-240.
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.
Romero-Calderón, R., Uhlenbrock, G., Borycz, J., Simon, A. F., Grygoruk, A., Yee, S. K., Shyer, A., Ackerson, L. C., Maidment, N. T., Meinertzhagen, I. A., Hovemann, B. T. and Krantz, D. E. (2008). A glial variant of the vesicular monoamine transporter is required to store histamine in the *Drosophila* visual system. *PLoS Genet.* **4**, 1-13.
Ross, R. H., Jr and Monroe, R. E. (1972). β-alanine metabolism in the housefly, *Musca domestica*: studies on anabolism in the early puparium. *J. Insect Physiol.* **18**, 1593-1597.
Sarthy, P. V. (1991). Histamine: a neurotransmitter candidate for *Drosophila* photoreceptors. *J. Neurochem.* **57**, 1757-1768.
Strøman, P. (1974). Pyrimidine-sensitive wing mutants *withered (whd), tilt (tt)* and *dumpy (dp)*. *Hereditas* **78**, 157-168.
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.
Tamura, S., Nelson, H., Tamura, A. and Nelson, N. (1995). Short external loops as potential substrate binding site of gamma-aminobutyric acid transporters. *J. Biol. Chem.* **270**, 28712-28715.
Tsacopoulos, M., Veuthey, A. L., Saravelos, S. G., Perrottet, P. and Tsoupras, G. (1994). Glial cells transform glucose to alanine, which fuels the neurons in the honeybee retina. *J. Neurosci.* **14**, 1339-1351.
Van Gelder, R. N., Bae, H., Palazzolo, M. J., Krasnow, M. A. (1995). Extent and character of circadian gene expression in *Drosophila melanogaster*: identification of twenty oscillating mRNAs in the fly head. *Curr. Biol.* **5**,1424-1436.
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.
Wang, X., Wang, T., Jiao, Y., von Lintig, J. and Montell, C. (2010). Requirement for an enzymatic visual cycle in *Drosophila*. *Curr. Biol.* **20**, 93-102.
Wolff, T. and Ready, D. F. (1993). Pattern formation in the *Drosophila* retina. In *The Development of Drosophila Melanogaster* (ed. M. Bate and A. Martinez Arias), pp. 1277-1325. Plainview, NY: Cold Spring Harbor Laboratory Press.
Wright, T. R. F. (1987). The genetics of biogenic amine metabolism, sclerotisation and melanisation in *Drosophila melanogaster*. *Adv. Genet.* **24**, 127-222.