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Vesicular neurotransmitter transporters in *Drosophila melanogaster*

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Abstract

Drosophila melanogaster express vesicular transporters for the storage of neurotransmitters acetylcholine, biogenic amines, GABA, and glutamate. The large array of powerful molecular-genetic tools available in *Drosophila* enhance the use of this model organism for studying transporter function and regulation.

Introduction

Here we provide an overview of vesicular neurotransmitter transporters in *Drosophila* and include additional background for readers unfamiliar with *Drosophila* as a model system. Advantages of *Drosophila melanogaster* for the study of neurotransmitter transporters as well as other transporters include their low cost, a short lifespan, and an ever-growing array of powerful molecular-genetic tools (Bellen et al., 2010; Ugur et al., 2016).

As in mammals, *Drosophila* vesicular neurotransmitter transporters localize to the membranes of secretory vesicles and are responsible for transport and storage of neurotransmitters into the vesicle lumen (Blakely and Edwards, 2012). Additionally, like mammals, *Drosophila* vesicular transporters are required for the storage of monoamines in synaptic vesicles (SVs) as well as large dense core vesicles (LDCVs) which also store and release peptide neurotransmitters (Nassel, 2018) (Fig 1). Unlike mammals which express two vesicular monoamine (VMAT) and three vesicular glutamate transporter (VGLUT) genes, flies express a single ortholog of VMAT and VGLUT, as well as a vesicular GABA (VGAT) and vesicular acetylcholine (VAChT) transporter.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Drosophila VMAT (*dVMAT*) and *dVAChT* are members of the SLC18 subfamily of the major facilitator superfamily (MFS), while *dVGAT* and *dVGLUT* are members of the SLC32 and SLC17 subfamilies, respectively (Martin and Krantz, 2014). Another member of SLC17 has also been identified in mammals as a vesicular ATP or nucleotide transporter (VNUT) (Sawada et al., 2008); a possible VNUT ortholog is present in the fly genome but has not yet been characterized (Moriyama et al., 2017). In addition to orthologs of VMAT and VAChT, flies express another SLC18 member that is not present in mammals, portabella (prt) (Brooks et al., 2011) and, to date, its substrate remains unknown. Finally, a member of the SLC45 family of putative sugar transporters primarily responsible for proton-mediated sugar transport in mammals, was recently identified as a vesicular histamine transporter in the *Drosophila* visual system (Xu and Wang, 2019).

A Brief Fly Neurobiology Primer

Fly Life Cycle

Flies pass through several distinct developmental stages prior to adulthood including one day as an embryo (Hartenstein and Wodarz, 2013; Menon et al., 2013), five days through three successive larval stages known as instars and five days as pupae. Vesicular transporters are generally expressed beginning in the late embryonic stage as the nervous system matures and neurons differentiate from precursor cells. Fly neurons support action potentials and calcium-mediated release at the nerve terminal similar to mammals. Unlike mammals, however, most terminals in the fly possess a characteristic presynaptic specialization known as a T-bar, around which SVs cluster. By contrast, LDCVs do not cluster near T-bars and fewer T-bars are found in terminals that contain primarily LDCVs (Atwood et al., 1993; Jia et al., 1993; Karsai et al., 2013).

In flies and other invertebrates, neuronal somata and processes are generally localized to stereotypic positions, both centrally and in the periphery. In the embryo and larva, the central nervous system includes the segmented ventral nerve cord and the bulbous, anterior structure variously referred to as “the larval brain” or the “fused subesophogeal and supraesophageal ganglia.” (Fig 2A).

During pupation, the nervous system is completely remodeled in parallel to the reorganization of the body plan from larva to adult. A variety of excellent resources describing adult fly neuroanatomy are available online including http://www.virtualflybrain.org/site/vfb_site/overview.htm. A simplified overview is shown in Fig 2. Major structures serving sensory functions within the adult brain include the optic ganglia (composed of the lamina, medulla, lobula and lobula plate) (Melnattur and Lee, 2011) and the antennal lobes which receive olfactory input from the antenna and maxillary palp (Masse et al., 2009; Mu et al., 2012; Wilson, 2013). The antennal lobes (roughly analogous to the mammalian olfactory bulb), and comprising cholinergic projection neurons, innervate the mushroom bodies (MBs) (functionally analogous to the mammalian hippocampus), a structure required for learning, memory and the integration of sensory information(Cognigni et al., 2018; Guven-Ozkan and Davis, 2014; Heisenberg, 2003; Iniguez et al., 2013; Ito et al., 1998; Kahsai and Zars, 2011). Additional morphologically distinct and functionally important structures in the adult central brain include the fan shaped body and the ellipsoid

body; both are elements of the central complex, which is associated with coordination of movement and other behaviors (Lebestky et al., 2009; Liu et al., 2012; Seelig and Jayaraman, 2013; Strauss, 2002; Ueno et al., 2012)

Fly Neurotransmitters

Drosophila use many of the same neurotransmitters as mammals including the monoamines dopamine (DA), serotonin (5HT) and histamine (Monastirioti, 1999). Histamine has been studied extensively in insects because of its role in the visual system and is synthesized by the enzyme histidine decarboxylase (Burg et al., 1993).

Unlike mammals, flies and other insects do not appear to synthesize more than trace quantities of the adrenergic neurotransmitters noradrenaline and adrenaline. Rather, two structurally similar molecules, tyramine and octopamine (OA), serve as the major adrenergic transmitters in *Drosophila* (Borowsky et al., 2001; Roeder, 2005). Their biosynthesis requires the initial conversion of tyrosine to tyramine by Tyrosine decarboxylase (Tdc) (Cole et al., 2005) (Fig 3). Hydroxylation at the β carbon catalyzed by Tyramine β Hydroxylase (TβH) converts tyramine to OA (Monastirioti et al., 1996) (Fig 3). Hydroxylation of the β carbon to generate OA is analogous to the conversion of DA to NE in mammals. As in mammals, flies synthesize DA via Tyrosine Hydroxylase (TH) and DOPA Decarboxylase (DDC) (Fig 3) (Birman et al., 1994). Also, like mammals, *Drosophila* synthesize GABA and acetylcholine (ACh) via glutamate via Glutamic Acid Decarboxylase (GAD) (Jackson et al., 1990) and Choline Acetyl Transferase (ChAT) respectively (Kitamoto et al., 1998). The genomic organization of the cholinergic locus is remarkably conserved, with the vesicular acetylcholine transporter (VAChT) contained within an intron of ChAT in flies as well as mammals (Kitamoto et al., 1998).

Both glutamate and ACh act as excitatory transmitters in the fly, but unlike mammals, glutamate rather than ACh is released at the fly neuromuscular junction (NMJ) (Jan and Jan, 1976). In addition, glutamate also functions as an inhibitory transmitter in flies through activation of a glutamate-gated chloride channel (Liu and Wilson, 2013; Rohrbough and Broadie, 2002). GABA appears to function strictly as an inhibitory transmitter in the fly (Lee et al., 2003).

Neurotransmitter Catabolism

Catabolism of neurotransmitters in *Drosophila* has, in general, received less attention than analogous processes in mammals, but in some instances, may be more dependent on conjugation than oxidation. For example, the enzyme ebony conjugates DA to β-alanine in the fly visual system (Suh and Jackson, 2007). Histamine also undergoes conjugation by ebony in a complex process that, at least in the visual system, involves transport of metabolites in and out of glia, analogous to glutamate recycling in mammals (Edwards and Meinertzhagen, 2010). (Fig 4). Histamine that is released from fly photoreceptors is taken up by glia by an unknown transporter. Within glial cells, ebony conjugates histamine to β-alanine to generate carcinine (Borycz et al., 2002; Richardt et al., 2002). Carcinine is then transported out of glia via an unknown mechanism and taken up into photoreceptors by the

recently defined Carcinine Transporter (CarT), a member of the SLC22 family of the MFS (Chaturvedi et al., 2016; Stenesen et al., 2015; Xu et al., 2015). It is reconverted into histamine in neurons by the cysteine peptidase tan (True et al., 2005; Wagner et al., 2007). Additional cells in the visual system may also contribute to histamine recycling and metabolism (Borycz et al., 2012; Romero-Calderon et al., 2008).

Flies do not appear to express an ortholog of the enzyme monoamine oxidase (MAO) which, in mammals, mediates the degradation of serotonin, dopamine and noradrenalin (Roelofs and Van Haastert, 2001). However, biochemical studies have demonstrated oxidative activity in fly homogenates and it is possible that oxidation plays a role in the degradation of some neurotransmitters in flies as in mammals (Chaudhuri et al., 2007; Wang et al., 2011; Yellman et al., 1997).

Expression of the plasma membrane serotonin and DA transporters in aminergic neurons suggests that, like mammals, serotonin and DA are likely to be recycled in presynaptic release sites (Bang et al., 2011; Giang et al., 2011). By contrast, plasma membrane GABA and glutamate transporters are expressed in *Drosophila* glia indicating that, as in mammals, glia likely play a prominent role in recycling these neurotransmitters (Neckameyer and Cooper, 1998; Parinejad et al., 2016; Seal et al., 1998; Soustelle et al., 2002; Stork et al., 2014).

Drosophila Vesicular Neurotransmitter Transporters

dVAChT

VAChT is widely expressed throughout the fly CNS, and more recently, has been shown to be expressed in the fly mushroom bodies (Barnstedt et al., 2016; Boppana et al., 2017; Pankova and Borst, 2017). As noted above, the mushroom bodies are critical for learning and memory in insects and are composed of several thousand intrinsic Kenyon cells (KCs). Earlier ultrastructural data on KC dendrites suggested that afferent processes, but not the KCs themselves, were cholinergic (Yasuyama et al., 2002). By contrast, more recent work using antibodies to dVAChT as well as epitope tagging of the endogenous VAChT locus have clearly shown that a large population of KCs can store and release ACh (Barnstedt et al., 2016; Boppana et al., 2017; Pankova and Borst, 2017). The functional importance of ACh release from KCs was demonstrated in multiple ways including the response of post-synaptic mushroom body output neurons to ACh, memory deficits following RNAi-mediated knock down of dVAChT in KCs and blockade of KC output with cholinergic drugs (Barnstedt et al., 2016).

In addition to RNA interference (RNAi) knock down studies (Barnstedt et al., 2016), both overexpression and genetic mutations in the endogenous *dVAChT* locus have been used to investigate its function. VAChT overexpression in *Drosophila* leads to reduced lifespan, age-dependent defects in locomotion as well as severe deficits in learning and memory (Showell et al., 2020). The findings on cognitive impairment mirrors prior studies in mice (Kolisnyk et al., 2013) and indicate that adverse effects may be associated with an increase in VAChT activity.

dVAChT loss of function mutants include *dVAChT*^{1} which is embryonic lethal and (Showell et al., 2020)putative null, and the weaker, "hypomorphic" allele *dVAChT*^{2}. *dVAChT*^{2} survives through the second larval stage but locomotes slower than wild type animals (Kitamoto et al., 2000), consistent with the identified role for ACh in the larval locomotive circuit (Baines, 2003; Malloy et al., 2019). Heterozygous *dVAChT* mutants survive to adulthood, but show subtle neuronal defects (Kitamoto et al., 2000). In particular, electrophysiological analysis of adult *dVAChT* heterozygotes suggests that during periods of sustained vesicle release, at least one circuit in the adult CNS fails to maintain normal levels of ACh release (Kitamoto et al., 2000). One mechanism underlying this change could be reduced numbers of VAChT molecules on each SV (Prado et al., 2013). In addition, it is likely that under conditions of reduced VAChT expression in both flies and mammals, some vesicles are likely to be devoid of VAChT or any luminal ACh (Prado et al., 2013). The contribution of each mechanism to the phenotype of specific mutants may vary depending on the rate of SV recycling and as well as the mechanism(s) responsible for determining when a vesicle is "full" (Williams, 1997).

Previous models proposed to determine when a vesicle is full include a static "set point" model and a more dynamic equilibrium model in which the balance between uptake and leakage regulates loading (Williams, 1997). The equilibrium model predicts that changes in the expression of vesicular transporters – the molecules that mediate loading – might change the amount of transmitter in each vesicle. In one recent study, increased levels of VAChT driven by overexpression of a transgene failed to show an increase in the amplitude of miniature electrophysiological potentials ("minis") which represent the post-synaptic response to a single SV (Cash et al., 2016). This was interpreted as supporting a set point model in which each vesicle contains fixed amount of neurotransmitter, at least in the central cholinergic synapses that were examined (Cash et al., 2016). However, in other circuits and systems, changes in vesicular transporter expression have been suggested to modify vesicular neurotransmitter content (Daniels et al., 2004; Lima et al., 2010; Song et al., 1997). Together, these data suggest the possibility that perhaps only a subset of neurons have the ability to increase (or decrease) the complement of vesicular transporters on each vesicle and thereby increase vesicle loading.

Another variable contributing to loading SVs is the intrinsic activity of each individual transporter. Further structure-functional analysis of dVAChT has demonstrated that, even if there is a set point in some cholinergic vesicles in the fly, it might be reset by altering transporter activity. This was recently shown by manipulating a polyglutamine region in the C-terminal cytoplasmic tail of dVAChT (Vernon et al., 2019). Wild type VAChT contains thirteen glutamines at this site and addition of one glutamine reduced both mini frequency and amplitude; conversely, a reduction from thirteen to twelve glutamines increased vesicle loading (Vernon et al., 2019). Since the polyglutamine repeat is restricted to insect species, this mechanism would not extend to mammalian VAChT. It nonetheless represents an interesting example of mechanisms that may regulate vesicular transporter activity and vesicle loading. Since the polyglutamine region in dVAChT is not directly involved in transport, some form of intramolecular interaction may be required. This could conceivably involve sites similar to those required for the G-protein mediated regulation of mammalian VMAT (Ahnert-Hilger et al., 1998; Brunk et al., 2006).

In an additional structure function study of dVAChT by the same group, a Y49N mutation identified as conferring insecticide resistance was shown to increase spontaneous mini frequency but dampen trains of evoked release (Vernon et al., 2018). The two phenomena might be causally related since an increase in the spontaneous release of vesicles could deplete the pool available for evoked release. Since Y49 is found within a putative trafficking motif (Vernon et al., 2018), it is tempting to hypothesize that the increase in minis could be caused by an alteration in VAChT trafficking. Indeed, increased trafficking to SVs might increase the number of vesicles that contain a single VAChT or possibly increase the number of VAChT molecules per SV.

dVGLUT

dVGLUT is expressed in all glutamatergic neurons in the larva and adult fly including the glutamatergic motor neurons innervating the NMJ (Daniels et al., 2004; Daniels et al., 2008). The membrane topology of dVGLUT has been examined using a series of epitope tags predicted to reside on the luminal and cytosolic loops between predicted transmembrane domains as well as at the N- and C-termini (Fei et al., 2007). Deletion of the cytosolic C-terminus abrogates lethality caused by dVGLUT over-expression, clearly implicating an important functional role for this domain including trafficking of the transporter to SVs (Grygoruk et al., 2010). These data are consistent with the presence of critical trafficking motifs that reside in the C-termini of mammalian VGLUTs (Foss et al., 2013; Voglmaier et al., 2006). However, the baseline localization of dVGLUT to SVs appears to be surprisingly unaffected by deletion of the C-terminus (Grygoruk et al., 2010) and studies of mammalian VGLUT trafficking have identified trafficking motifs in both the N and C termini (Foss et al., 2013; Voglmaier et al., 2006). It is possible that the contribution of specific domains to VGLUT trafficking could vary between cell types or species.

Similar to studies of *Drosophila* and mammalian VAChT, miniature end plate potentials have been used to examine neurotransmitter content in *dVGLUT* mutants (Daniels et al., 2006). In mutant flies expressing reduced levels of dVGLUT, mini frequency was also reduced, suggesting that some exocytosed SVs lacked dGVLUT and were therefore “empty.” More surprisingly, the size of the remaining minis, an indication of SV filling, was not altered in the mutant. These data suggest that a reduced number of VGLUT molecules, and perhaps only one, may suffice to fill a vesicle under some conditions (Daniels et al., 2006). However, using other techniques, recent estimates of VGLUT copies per vesicle in wild type mammalian preparations have ranged from 4 to 14 (Mutch et al., 2011; Takamori et al., 2006).

Over-expression of dVGLUT at high levels using relatively strong GAL4 drivers is larval lethal (Daniels et al., 2011). Over-expression using a weaker driver allows survival through adulthood allowing the study of the adult phenotype (Daniels et al., 2011). Interestingly, the surviving adults show large lacunae in their CNS, possibly the result of glutamate-mediated excitotoxicity (Daniels et al., 2011). Consistent with the observation that dVGLUT over expression causes functional changes in SV homeostasis, electrophysiological studies of dVGLUT over-expression in larva show an increase in quantal size, as well as large spontaneous events at the larval NMJ (Daniels et al., 2004; Daniels et al., 2011), a

phenotype similar to that seen with over-expression of vesicular transporters in mammals (Edwards, 2007; Wilson et al., 2005).

dVMAT

dVMAT encodes at least two splice variants, dVMAT-Λ and -B, which differ at their C-termini (Greer et al., 2005). dVMAT-A is expressed in both larvae and adults in all dopaminergic, serotonergic and octopaminergic cells (Chang et al., 2006; Greer et al., 2005). Selective rescue of dVMAT function in either individual or multiple aminergic systems within a dVMAT null mutant background was used to determine the respective contributions of dopaminergic, serotonergic and/or octopaminergic/tyraminergic neurons to amine-dependent behaviors (Chen et al., 2013). In addition to supporting known roles of each neurotransmitter in behavior, this analysis provides a useful approach to define new roles that might be difficult to study using biosynthetic mutants (Adamo et al., 1995; Alekseyenko et al., 2010; Bacon et al., 1995; Fox et al., 2006; Koon et al., 2011; Lee et al., 2009; Monastirioti et al., 1996).

In contrast to dVMAT-A and mammalian VMATs, dVMAT-B is expressed in a subset of glia rather than neurons (Romero-Calderón et al., 2008). Its function in the optic lobes may include storage of histamine or its metabolite carcinine (Romero-Calderón et al., 2008). Although mammalian glia likely take up amines via low affinity mechanisms (Dahlin et al., 2007; Yoshikawa et al., 2013), the expression of a specific amine transporter in glia is unusual and further studies of dVMAT-B may help determine the role of glia in the recycling of histamine and perhaps other biogenic amines.

dVMAT mutants show a number of behavioral deficits consistent with the loss of exocytotic amine release in the nervous system (Chen et al., 2013; Simon et al., 2009). Similar to mouse knockouts, mutant tissue levels of amines are dramatically reduced, presumably due to the degradation of amines that are not sequestered in secretory vesicles (Fon et al., 1997; Simon et al., 2009). Under standard fly culture conditions, loss of *dVMAT* is lethal, but reducing the density of the culture can increase viability, in at least some genetic backgrounds, thus allowing for use of *dVMAT* mutants in behavioral assays. *dVMAT* mutants show a dramatic decrease in baseline larval locomotion (Simon et al., 2009), mirroring previously demonstrated effects of dVMAT blockade by reserpine in flies with wild type dVMAT expression (Pendleton et al., 2000).

Both mutation of *dVMAT* and pharmacologic inhibition with reserpine cause increased sleep (Nall and Sehgal, 2013). In contrast, amphetamines decrease sleep in flies (Andretic et al., 2005), presumably via release of DA (Pizzo et al., 2013). In each case, changes in sleep were distinguished from changes in locomotion using previously established criteria (Hendricks et al., 2000; Shaw et al., 2000). The circuits underlying these effects remain unclear but could potentially be generally relevant to the behavioral effects of stimulants in mammals.

“Genetic rescue” experiments in which *dVMAT* mutant transgenes were expressed in a *dVMAT* null mutant background have provided a convenient method for analyzing the effects of the mutant alleles. Mutation of signals in the C-terminus of dVMAT-A blocks endocytosis in cultured cells and disrupts trafficking to SVs when expressed *in vivo*

(Grygoruk et al., 2010). This also leads to a corresponding increase in the localization of dVMAT to LDCVs (Grygoruk et al., 2014). Genetic rescue with dVMAT trafficking mutants differentially affected subsets of aminergic circuits, with some showing profound deficits and others showing little or no change relative to wild type (Grygoruk et al., 2014). Further studies of the circuits disrupted by dVMAT trafficking mutants may shed light on the function of amine release from SVs versus LDCVs, a topic that remains poorly understood. Additional genetic rescue experiments in the *dVMAT* null background have been used to visualize subpopulations of monoaminergic terminals labeled with fluorescent false neurotransmitters (FFNs). This combination of genetics with imaging and pharmacology enabled dissection of the mechanisms underlying the actions of amphetamines *in vivo* within whole intact fly brains (Aguilar et al., 2017; Freyberg et al., 2016). These studies also demonstrated that the tandem activities of both the *Drosophila* dopamine transporter (dDAT) and dVMAT are essential for the ability of amphetamines to redistribute DA out of the vesicle lumen into the cytoplasm and eventually out of the terminals – a key mechanism for these drugs' psychostimulant effects (Freyberg et al., 2016).

Flies over-expressing dVMAT in DA and 5HT cells show an increase in motor activity and a blunted behavioral response to cocaine (Chang et al., 2006). Likewise, administration of cocaine increases motor activity in the fly (Bainton et al., 2000; McClung and Hirsh, 1998; Torres and Horowitz, 1998). Similar to amphetamines (Andretic et al., 2005), over-expression of dVMAT in cells producing both 5HT and DA increases male courtship behavior (Chang et al., 2006). Importantly, the effects of dVMAT as well as dVGLUT over-expression demonstrate that the changes in neurotransmitter release seen with overexpression of vesicular transporters *in vitro* (Pothos et al., 2000; Song et al., 1997) can have significant downstream behavioral sequelae in an intact organism.

Over-expression of dVMAT as well as loss of function alleles have been used to explore *in vivo* the role of DA homeostasis in neurodegenerative processes relevant to Parkinson's disease (PD) (Lawal et al., 2010; Sang et al., 2007). DA has a high oxidative potential, in part through the formation of dopamine quinone, which may form adducts with DNA (Stokes et al., 1996; Stokes et al., 1999) and conjugate to proteins implicated in genetic forms of PD (Conway et al., 2001; Hastings et al., 1996; Van Laar et al., 2009). Thus, it has been suggested that an increase in VMAT activity may have neuroprotective effects (reviewed in (Guillot and Miller, 2009)). This has been tested *in vitro* in cell cultures using mammalian VMAT and dVMAT (Mosharov et al., 2009; Park et al., 2007), and *in vivo* in the fly (Inamdar et al., 2013; Lawal et al., 2010; Sang et al., 2007). Over-expression of dVMAT rescues the loss of DA neurons caused by either a genetic or chemical insult (Inamdar et al., 2013; Lawal et al., 2010; Sang et al., 2007). Conversely, loss of dVMAT activity increases the toxicity of both genetic and chemical insults to DA cells (Inamdar et al., 2013; Lawal et al., 2010; Sang et al., 2007). Similar to studies of VMATs in mammals (Caudle et al., 2007; Mosharov et al., 2009; Sulzer et al., 2000), these studies suggest the possibility that drugs which increase VMAT activity could show neuroprotective effects in models of PD. Accordingly, a mutant showing diminished levels of dVMAT expression (*i.e.*, a "functional hypomorph") was used to screen for drugs that might increase dVMAT activity or otherwise increase vesicular amine storage (Lawal et al., 2014). The results of this screen yielded several molecules that *a priori* would not be classified as aminergic but could

indirectly modify dVMAT activity (Lawal et al., 2014). Similar screens might be used to identify drugs that can directly or indirectly modify the activity of other transporters.

dVGAT

Like mammals, the *Drosophila* genome contains a single vesicular GABA transporter gene (*dVGAT*) (Fei et al., 2010). *dVGAT* appears to be expressed in all GABAergic neurons in fly larva since it precisely co-localizes with GABA in the ventral nerve cord, and is also expressed in most if not all adult GABAergic neurons (Enell et al., 2007; Fei et al., 2010). Mutation of *dVGAT* causes developmental lethality (Fei et al., 2010). To overcome this potential limitation, inducible expression of a *dVGAT* transgene has been used to restore dVGAT function during development and thereby allow adult behavioral studies. One adult phenotype demonstrated in the conditional rescue line was a surprisingly specific defect in the detection of small objects in the fly's visual field (Fei et al., 2010). Other aspects of reduced GABAergic signaling in this transgenic model remain to be explored.

It is not clear whether *Drosophila*, like mammals, use glycine as a neurotransmitter and whether dVGAT could also serve to store glycine. Since the structure of β-alanine is intermediate between glycine and GABA and mammalian and VGAT has been shown to transport β-alanine in vitro (Juge et al., 2013), it is also possible that dVGAT could play a role in β-alanine storage or metabolism in the fly. To date, such a possibility has not yet been tested. As noted above, conjugation of β-alanine to histamine is also required for histamine recycling in the fly (Borycz et al., 2002), and conjugation of β-alanine is important for DA metabolism in both the cuticle and the central nervous system (Suh and Jackson, 2007; Wright, 1987). This opens the door to further investigations of the intersections between dVGAT, histamine, and DA.

portabella

Drosophila and some other insects express *portabella*, an additional vesicular transporter that appears to be absent from mammalian genomes (Brooks et al., 2011; Lawal and Krantz, 2013). Similar to VAChT, *prt* is expressed in the KCs within the mushroom bodies (Brooks et al., 2011). Mutation of *prt* results in a peculiar defect in sexual behavior, characterized primarily by an inability of males to maintain their position during copulation (Brooks et al., 2011). The primary structure of the prt protein is most similar to dVMAT and it is possible that the substrate is also similar to known monoamines; however, the biosynthetic enzymes for DA, 5HT, OA and histamine are not expressed in KCs, suggesting that prt may transport a novel neurotransmitter (Brooks et al., 2011). Alternatively, it is possible that prt may act primarily to regulate the function of VAChT, similar to the proposed role of some other co-expressed vesicular transporters (see below).

LOVIT

In *Drosophila*, histamine functions as the primary neurotransmitter in photoreceptor cells (Nassel, 1999; Sarthy, 1991; Stuart et al., 2007). In mammals, VMATs are thought to be responsible for histamine storage but *dVMAT* is not expressed in fly photoreceptors (Chang et al., 2006). Recently, a member of the SLC45 family of putative sugar transporters, SLC45A2, has been implicated in vesicular histamine transport and storage, particularly in

the context of modulation of fly vision, and thus renamed Loss of Visual Transmission (LOVIT) (Xu and Wang, 2019). LOVIT is highly expressed in the terminals of photoreceptors and localized to SVs. Mutation of *lovit* resulted in loss of histamine in photoreceptor terminals, disrupting the response of postsynaptic neurons to light stimuli in adult flies and also ablating phototactic behavior (Xu and Wang, 2019). Conversely, the expression of LOVIT in the photoreceptors of *lovit* mutant flies restored the response of postsynaptic neurons to light stimuli. Together, these data indicate that LOVIT is responsible for vesicular storage and release of histamine in the photoreceptor cells of *Drosophila*. It is possible that LOVIT is also responsible for vesicular storage of histamine in other cells in the fly but this remains unclear.

Co-release of neurotransmitters and neuropeptides

In mammals, it is well established that neurons can co-release classical neurotransmitters and neuropeptides from LDCVs (Granger et al., 2017). A recent review summarized the colocalization of neuropeptides and neurotransmitters in fly neurons regulating behaviors such as sleep, olfaction, feeding and developmental processes (Nassel, 2018). In addition, a recent report suggested that a receptor protein tyrosine phosphatase Ptp4E may post-transcriptionally up-regulate neuropeptide content in dense core vesicles (Tao et al., 2019). Interestingly, Ptp4E deficiency was also reported to upregulate presynaptic dVMAT expression and activity at the same octopaminergic synapses (Tao et al., 2019). Future studies of this relatively simple circuit may be useful to explore mechanisms that may govern the storage and co-release both classical neurotransmitters and neuropeptides.

Co-expression of vesicular transporters

Multiple studies in both invertebrates and mammals indicate that more than one classical neurotransmitter can be stored and released from the same neuron. In most cases, this requires the co-expression of two vesicular transporters within the same cell (Granger et al., 2017; Hnasko and Edwards, 2011; Munster-Wandowski et al., 2016). In some cases, vesicular transporters may reside on the same vesicle; however, the context in which occurs remains an active topic of investigation and may vary across both synapses and cell types (Galvan and Gutierrez, 2017; Morales and Margolis, 2017; Silm et al., 2019).

In addition to co-release, co-localization on the same vesicle allows regulatory interactions between vesicular transporters (Munster-Wandowski et al., 2016). The molecular genetic tools available in the fly are well suited to explore this phenomenon. Similar to findings in mammals, dVGLUT is expressed in a subset of DA neurons in the adult fly central brain (Aguilar et al., 2017; Hnasko et al., 2012; Trudeau and El Mestikawy, 2018). Recent work has shown that depolarization of these cells causes an increase in the DA content of SVs immediately prior to exocytosis (Aguilar et al., 2017). The pH gradient across the vesicle membrane (ΔpH) is the primary driving force for dVMAT mediated loading of DA into SVs (Hnasko and Edwards, 2012), and dVGLUT was required to increase the ΔpH that was in turn responsible for increased DA loading and release (Aguilar et al., 2017). These results suggest a unique role for dVGLUT in tuning vesicular DA release to meet the demands of neuronal activity.

In addition to dVGLUT's role in the regulation of intraluminal SV pH, recent work has shown that following its localization to the plasma membrane during exocytosis, dVGLUT can modulate proton efflux from nerve terminals (Rossano et al., 2017). Indeed, dVGLUT's previously unrecognized ability to extrude protons may be critical for maintaining a stable cytoplasmic pH despite the movement of protons out of SVs required for dVMAT-dependent neurotransmitter transport into vesicles (Rossano et al., 2017).

dVGLUT expression in DA neurons also has been implicated in modulating their vulnerability to neurodegeneration. Heterologous over-expression of dVGLUT in DA neurons induced cell death in subsets of DA neurons including those innervating the central complex (Steinkellner et al., 2018). This phenomenon is analogous to experiments using mammalian DA neurons in which VGLUT2 expression was demonstrated to play a role in DA neuron vulnerability to neurotoxic insults (Shen et al., 2018; Steinkellner et al., 2018). Interestingly, recent work in mammals also showed that, although almost all DA neurons express VGLUT2 early in life, most repress its expression by adulthood with the exception of a relatively small population of DA neurons in the mouse ventral tegmental area (Steinkellner et al., 2018). It remains unclear whether a similar developmental repression of dVGLUT expression occurs in fly DA neurons. Nevertheless, these findings suggest that overriding the endogenous regulation via ectopic VGLUT expression is selectively toxic to adult DA neurons that normally repress or, at a minimum, are not equipped to express significant levels of VGLUT2 or dVGLUT.

Summary

Despite significant differences in anatomy, many of the molecular and cellular aspects of neurotransmitter storage in flies and mammals are similar. The abundance of molecular-genetic tools as well their low cost and short lifespan make *Drosophila* an attractive model for the study of vesicular transporters and their regulation.

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Highlights

- *Drosophila melanogaster* express orthologs of mammalian vesicular transporters
- *Drosophila* also express some vesicular transporters not found in mammals
- Mutants and transgenes available in *Drosophila* can be used to study transporter function

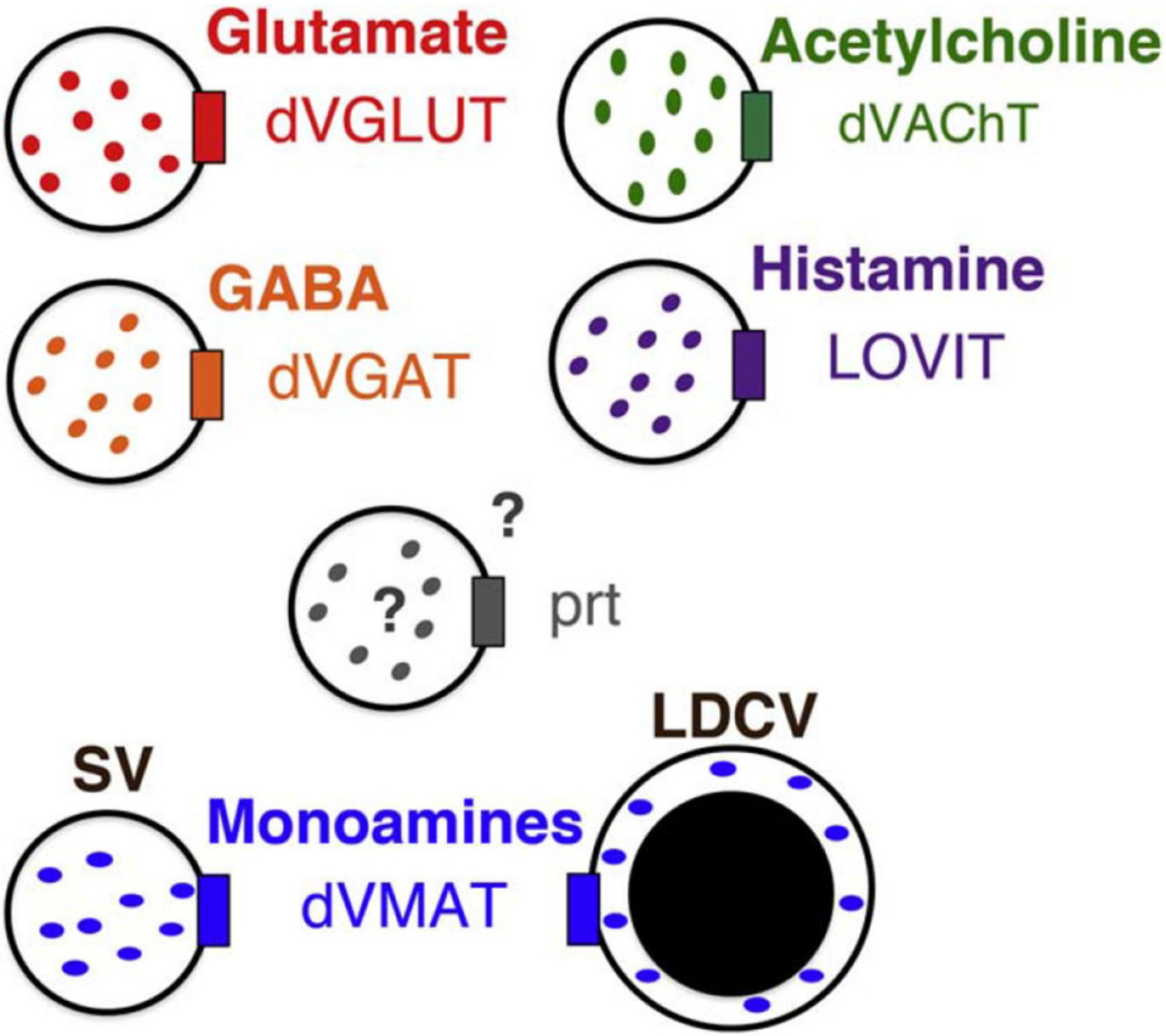

Fig 1. *Drosophila* Vesicular neurotransmitter transporters.

Vesicular neurotransmitter transporters expressed in the fly include one variant each of VGLUT, VGAT, VMAT and VAChT. VMAT localizes to both synaptic and dense core vesicles (SVs and LDCVs respectively) while VGLUT, VAChT and VACHT are primarily confined to SVs. Additional vesicular transporters that are expressed in flies but not mammals include the orphan transporter *portabella* (*prt*) and the histamine transporter, LOVIT.

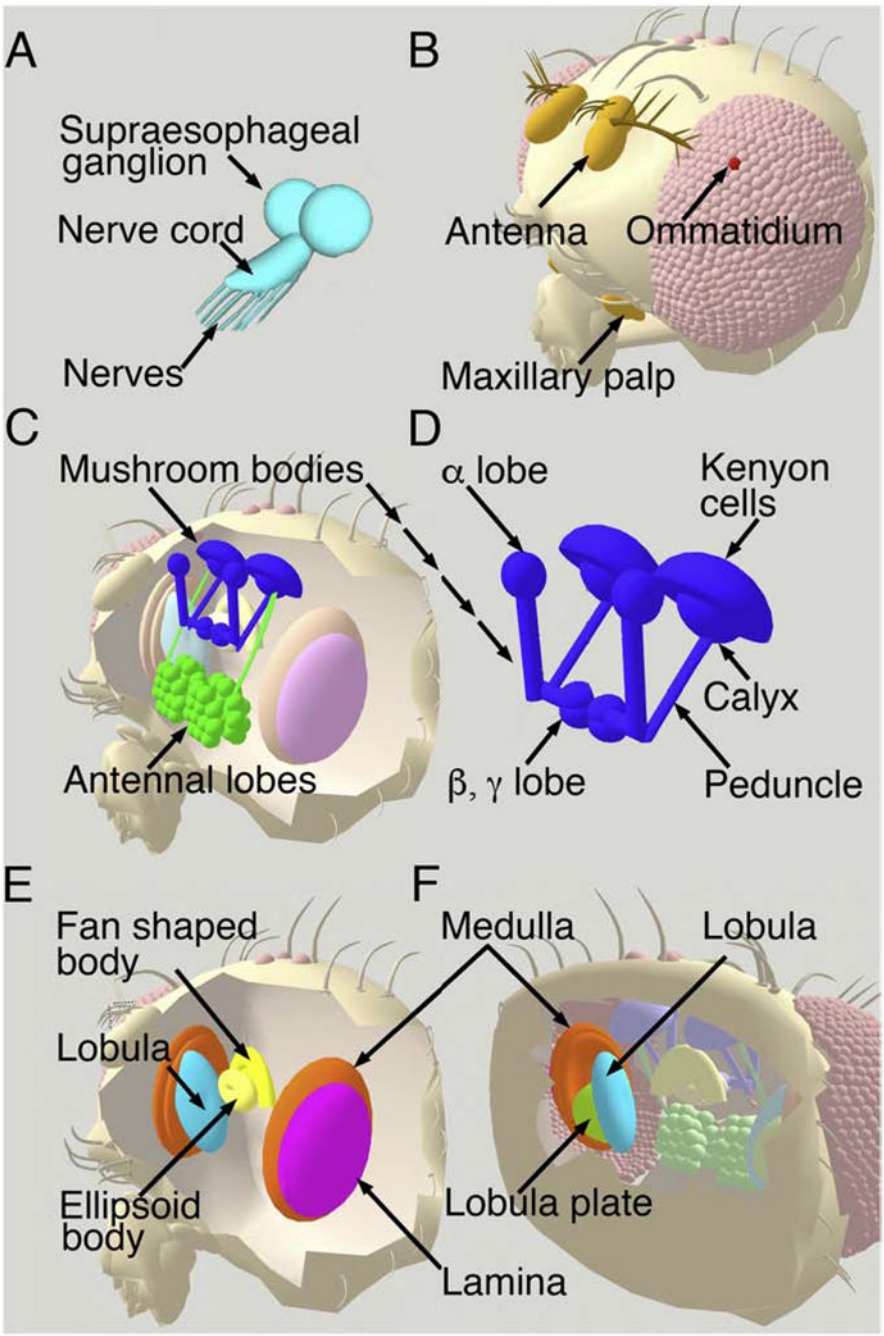

Fig 2. Basic neuroanatomy of larval and adult *Drosophila*.

A) The central nervous system of *Drosophila* larva is composed of the ventral nerve cord and the fused sub- and supra-esophageal ganglia, labeled here as the supraesophageal ganglion. It is sometimes loosely referred to as the brain or developing optic lobes. Nerve bundles emanating from the nerve cord contain both efferent processes from motoneurons as well afferents from sensory cells. The adult central nervous system includes neurons within the head capsule (B-F) and the thoracic nerve cord (not shown) as well as peripheral sensory neurons that send afferents into the CNS (not shown) and a loose network of neurons

associated with viscera (not shown). B) The external anatomy of the head capsule includes a large number of small and large bristles (micro and macrochaetae); only a few are shown. Both the maxillary palp and the distal portion of the antenna contain olfactory neurons that project to the antennal lobes. The retina of the eye is composed of several hundred clusters of photoreceptors known as ommatidia, each composed of eight individual photoreceptor cells. A single ommatidium is highlighted. C) Each of the two bilaterally symmetric antennal lobes (green) are composed of clusters of neurons arranged into glomeruli, represented here as spheres. Projections from the antennal lobes (green) innervate the mushroom bodies (blue) as well as regions of the central brain that are not shown. D) The mushroom bodies are composed of a dorsal “cap” containing the cell bodies of the Kenyon cells. The dendrites of the Kenyon cells make up the Calyx of the mushroom bodies and receive inputs from the antennal lobes. The Kenyon cell axons continue anteriorly and ventrally through the Peduncle and terminate in the vertically oriented α lobes and the horizontally oriented β and γ lobes. Front (E) and back (F) views of the head show the optic ganglia including the lamina (pink), medulla (orange), lobula (light blue) and lobula plate (green). Cells in the first two optic ganglia--the lamina and medulla-- receive input from photoreceptor cells and send processes to the third and fourth optic ganglia--the lobula and lobula plate-- for higher order processing. Two elements of the central complex are shown: the fan shaped body and ellipsoid body (both yellow).

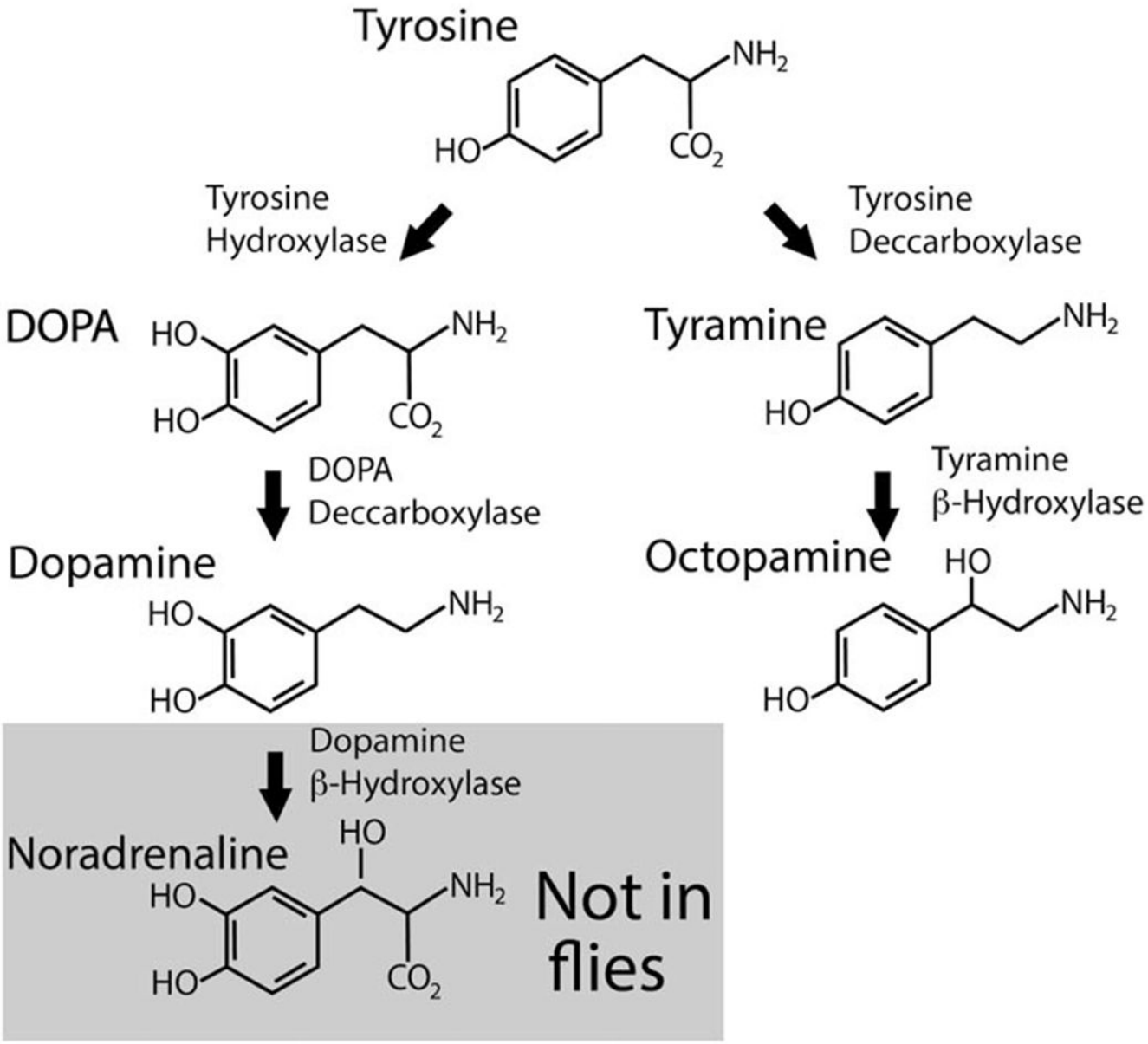

Fig 3. Biosynthesis of dopamine, tyramine and octopamine.

The major adrenergic neurotransmitters in the fly are tyramine and octopamine. Flies synthesize tyramine and octopamine using tyrosine decarboxylase to first convert tyrosine to tyramine. Tyramine β-Hydroxlase generates octopamine from tyramine. This is distinct from the evolutionarily conserved pathway used by both flies and mammals to generate dopamine via the successive actions of Tyrosine hydroxylase and Dopa decarboxylase (DDC) also known as Aromatic amino acid decarboxylase (AADC).

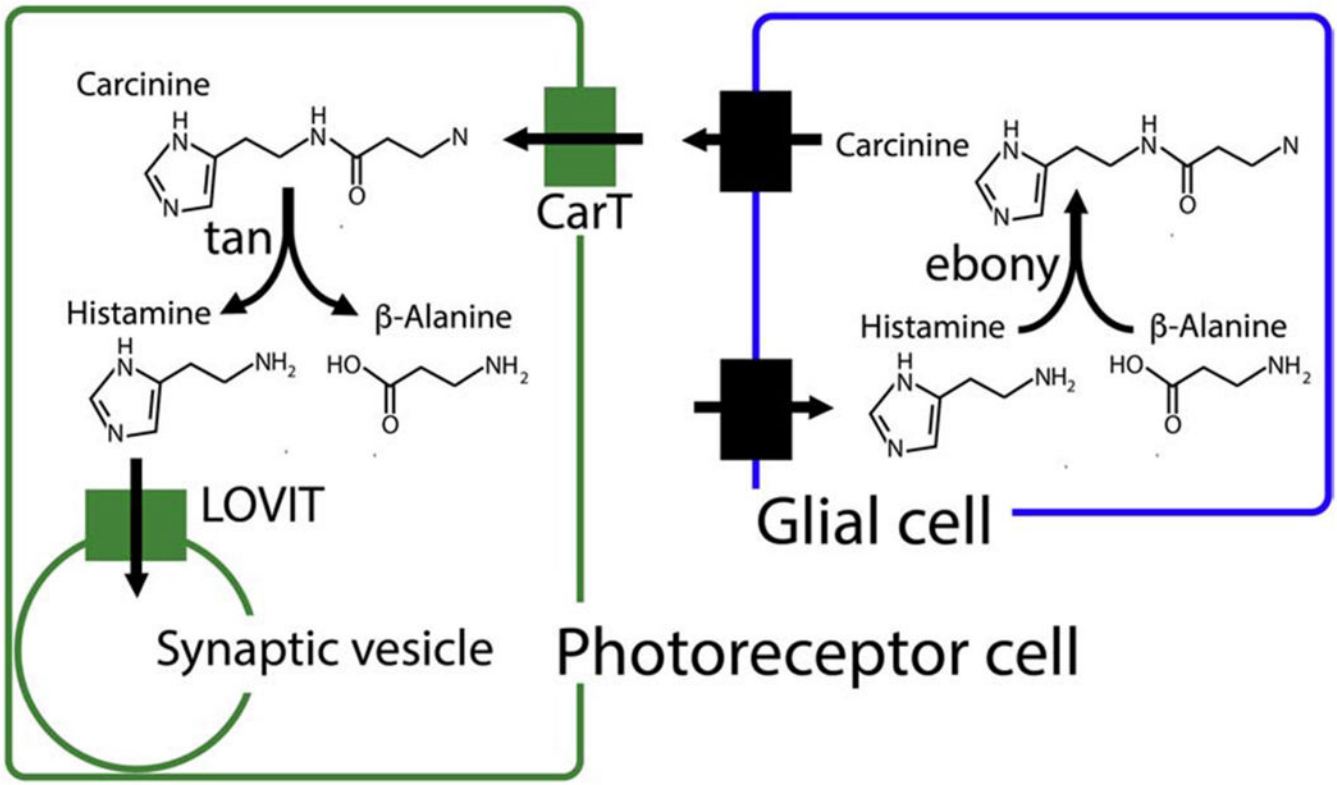

Fig 4. Histamine recycling in the fly visual system.

Histamine is stored in synaptic vesicles within photoreceptor cells by the vesicular transporter LOVIT. After exocytotic release, histamine is taken up by glia via an unknown transporter and conjugated to β-alanjne by the enzyme ebony to generate carcinine. Carcinine leaves glia via an unknown mechanism and is transported into photoreceptor cells by CarT, where it is converted to histamine by the peptidase tan.