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Regulation of Transmission at Monoaminergic Neurons: A Synapse-to-Circuit Study in Drosophila

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Regulation of Transmission at Monoaminergic Neurons:

A Synapse-to-Circuit Study in *Drosophila*

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in Neurobiology

by

Audrey Chen

2012

ABSTRACT OF THE DISSERTATION

Regulation of Transmission at Monoaminergic Neurons:

A Synapse-to-Circuit Study in *Drosophila*

by

Audrey Chen

Doctor of Philosophy in Neurobiology

University of California, Los Angeles, 2012

Professor David E. Krantz, Co-chair

Professor Felix E. Schweizer, Co-chair

The *Drosophila* vesicular monoamine transporter (DVMAT) regulates the loading and

storage of monoaminergic transmitters in secretory vesicles, and proper localization to vesicles is

required for the exocytotic release of neurotransmitters such as dopamine and serotonin. By

genetically modifying the availability of vesicular monoamine transporters in specific

monoamine neurons, I examine amine requirements for aspects of behavior and interactions

between aminergic systems. I demonstrate that some behaviors rely predominantly on

octopaminergic circuits with little apparent input from either serotonin or dopamine. In contrast,

other behaviors can be rescued by expressing DVMAT in octopaminergic or dopaminergic

neurons, suggesting potentially redundant circuits. Rescue of major aspects of adult locomotion

and startle behavior required octopamine, but complementary roles were observed for serotonin

and dopamine. Interestingly, adult circadian behavior could not be rescued by expression of

DVMAT in a single subtype of aminergic neurons, but required at least two systems, suggesting

the possibility of unexpected cooperative interactions. Studies using the temperature-sensitive

GAL80 inducible transgene system demonstrate that the temporal demand for DVMAT yields a

large degree of flexibility with causes still left unclear. In order to understand the cellular

mechanisms which regulate monoamine release, my work also examines motifs involved in

trafficking vesicular monomamine transporters to the axon terminal and appropriately on

synaptic vesicles during rounds of exo- and endocytosis. The C-terminus of DVMAT encodes a

tyrosine-based motif (YXXØ) flanked between a di-leucine motif and downstream acidic

residues that have been shown to be involved with endocytosis, sorting to synaptic vesicles and

maturation of large dense core vesicles *in vitro.* I employ fast-capture, *in vivo* real-time imaging

of pH-sensitive pHlourins to track wild-type and mutant DVMAT in intact circuits. My findings

suggest that the C-terminus contains trafficking motifs that slow endocytosis kinetics and

dampen the presynaptic neuron's ability to recruit vesicles to the plasma membrane. Moreover,

the previously described Δ3 deletion disrupts VMAT trafficking to the axon terminal. Combined

with results showing that behaviors are differentially sensitive to mutations, these data are among

the first to demonstrate that mislocalization of a synaptic protein may preferentially affect

specific neuronal circuits and argue that behaviors require varying stringency levels for regulated

neurotransmitter release.

The dissertation of Audrey Chen is approved.

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2012

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PUBLICATIONS AND PRESENTATIONS

Chen A, Ng F, Lebestky T, Grygoruk A, Djapri C, Zaveri HA, Mehanzel F, Najibi R, Seidman G, Lawal HO, Kelly RL, Murphy NP, Ackerson LC, Maidment NT, Jackson FR, Krantz DE. Selective neuronal restoration of vesicular monoamine transporter function uncovers coordinated, complementary and dispensable roles for dopamine, octopamine and serotonin in *Drosophila melanogaster*. (Submitted to *Genetics*, In Review)

Chen, A. (February 2011). *Probing Synaptic Vesicle Dynamics in a Drosophila Vesicular Monoamine Transporter (dVMAT) Trafficking Mutant*. Talk presented at the Synaptic Biophysics and Excitable Cell Physiology: A Symposium in Honor of Yoshi Kidokoro, University of California, Los Angeles, California.

Grygoruk A, Fei H, Daniels RW, Miller BR, Chen A, DiAntonnio A, Krantz DE. 2010. Vesicular neurotransmitter transporter trafficking *in vivo*: moving from cells to flies. *Fly* 4(4):302-305.

Fei H, Chow DM, Chen A, Romero-Calderon R, Ong WS, Ackerson LC, Maidment NT, Simpson JH, Frye MA, Krantz DE. 2010. Mutation of the Drosophila vesicular GABA transporter disrupts visual figure detection. *J Exp Biol* 213: 1717-1730.

Chen, A. (April 2009). *Genetic Dissection of Complex Behaviors in Drosophila Mutants of the Vesicular Monoamine Transporter (VMAT)*. Talk presented at the UCLA Neurobiology Departmental Retreat, Malibu, California.

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CHAPTER ONE:

General Introduction

Monoaminergic circuits regulate a range of behaviors, including sleep, appetite, reward, and attention. Both invertebrate and mammalian behaviors undergo extensive regulation by monoamine neurotransmitters released from a relatively small number of neurons (Reviewed in BARRON *et al.* 2010; BROMBERG-MARTIN *et al.* 2010; HAENISCH and BÖNISCH 2011; JOSHUA *et al.* 2009; SARA 2009; SCHULTZ 2010; SHOHAMY and ADCOCK 2010). In mammals, serotonin (5-HT), dopamine (DA), noradrenaline (NE), adrenaline and histamine modulate these circuits (BARNES *et al.* 2011; DAUBERT and CONDRON 2010; HAENISCH and BÖNISCH 2011; SARA 2009; SCHULTZ 2010; SHOHAMY and ADCOCK 2010; SOUTHWICK *et al.* 2005). In invertebrates, two additional tyrosine derivatives, octopamine and tyramine, act as monoaminergic neurotransmitters. Structurally similar to NE, octopamine differs only by a hydroxyl group at position 3 in the phenol ring, permeates invertebrate physiology in fairly high concentrations, and appears to modulate every physiological process in invertebrates (AXELROD and SAAVEDRA 1977; HARDIE and HIRSH 2006; ROEDER 1999). Although low levels of NE have been found in most invertebrates, few studies have examined the proposed NE receptors or reported a physiological role of the neurotransmitter in invertebrates (COON and BONAR 1986; MARDER 2007). In invertebrates, octopamine appears to play a role in the 'flight-or-fight' response and other behaviors traditionally attributed to NE in mammals (ADAMO 2008); therefore, OA holds both structural and functional similarity with NE. Along with 5-HT, DA, histamine, and tyramine, octopamine (OA) modulates circuits (BARRON *et al.* 2010; BLENAU and BAUMANN 2001; COLE *et al.* 2005; DAUBERT and CONDRON 2010; FAROOQUI 2007; HARDIE *et al.* 2007; MONASTIRIOTI *et al.* 1996; ROEDER 2005; WADDELL 2010).

Vesicular monoamine transporters are required for neurotransmission at monoaminergic synapses

All synapses which transmit signals via monoamines possess vesicular monoamine transporters (VMATs) to fill vesicles with monoamines. In contrast to plasma membrane transporters which sit at the plasma membrane and function to terminate the action of released neurotransmitter, vesicular monoamine transporters transverse vesicular membranes and function to package monoaminergic transmitters for regulated release. In mammals, two isoforms, VMAT1 and VMAT2, have been identified and characterized (ERICKSON *et al.* 1996; LIU *et al.* 1992; PETER *et al.* 1995). VMAT2 is expressed in amineric neurons in the central nervous system, while VMAT1 is primarily expressed in neuroendocrine cells. In *Drosophila,* the genome contains one VMAT (*dVMAT*) gene, which is alternatively sliced to produce two splice variants (dVMAT-A and B) with the former holding homology with the mammalian neural isoform VMAT2 (GREER *et al.* 2005).

The extent of neurotransmitter transport into a vesicle can be influenced by a number of factors, including the H^+ electrochemical gradient, cytosolic concentration of neurotransmitter, transporter activity and nonspecific leakage from secretory vesicles (EDWARDS 2007). For VMAT, transport relies predominantly on the chemical proton gradient rather than the electrical gradient. Once packaged into a vesicle, neurotransmitters may require counterions to release them from the secretory granule matrix inside vesicles and diffuse into the synaptic cleft when vesicles fuse to the plasma membrane during rounds of exocytosis (RAHAMIMOFF and FERNANDEZ 1997).

In the absence of VMAT activity, central vesicular monoamine transporter 2 (VMAT2) knockout mice display reduced ability to store and release monoamine transmitters (FON *et al.* 1997; TAKAHASHI *et al.* 1997; WANG *et al.* 1997). When VMAT is overexpressed, quantal size and frequency of events evoked by stimulation are increased, resulting in increased neurotransmitter released into the synaptic cleft, and cytoplasmic dopamine levels are decreased (COLLIVER *et al.* 2000; POTHOS *et al.* 2000).

Revisiting the quantal nature of chemical transmission

In the 1950s, Bernard Katz and colleagues established the quantal nature of chemical transmission by showing that end-plate potentials (EPP) at the frog neuromuscular junction (NMJ) were composed of small all-or-none units identical in size with the spontaneous miniature EPPs (DEL CASTILLO and KATZ 1954; FATT and KATZ 1952). Corroborating with these electrophysiological findings, electron micrograms illustrating membrane-bound vesicles at nerve terminals suggested the structural correlate of these "quanta" (see Palade and Palay (1954) and accompanying articles). Studies that showed that synaptic vesicle fusion coincides temporally with quantal release (TORRI-TARELLI *et al.* 1985) and one quantum corresponds to one vesicle (HURLBUT *et al.* 1990) further consolidated our current dogma. However, Katz's original definition of the quantum did not imply that it held a fixed size. The notion that each quantum was a fixed size likely developed as a consequence of emphasis on the mean quantal size and the accompanying finding that calcium controls the probability of release rather than the size of the quantum. As a result, studies on synaptic function have focused on the probability and number of quanta released, assuming that the size of the quantum was fixed.

Recently, several groups have shown that quantal size, the postsynaptic response to neurotransmitter release from a single quantum, is flexible and susceptible to presynaptic modifications. Studies in mammalian slices have shown that artificial perturbation of presynaptic parameters – the electrochemical proton gradient responsible for driving neurotransmitter loading into vesicles, cellular glutamate concentrations, and vesicular neurotransmitter transporter expression – can alter quantal size, indicating changes in vesicular content (FREMEAU *et al.* 2004; ISHIKAWA *et al.* 2002; WOJCIK *et al.* 2004). In addition, the field has recently shown that vesicles may alter the number of vesicular transporters in a manner that is physiologically relevant. It has been demonstrated that endogenous regulation of quantal size occurs in order to support larger synaptic workloads (STEINERT *et al.* 2006). During an intermediate "*phase-I* of experience-dependent potentiation" (40-80 min of high crawling activities), active *Drosophila* larvae recruit larger vesicles than their less-active peers. During development, neurons alter quantal size by controlling vesicular transporter expression to facilitate activity-dependent plasticity during development (WILSON *et al.* 2005). If quantal size can be adjusted using presynaptic mechanisms, a neuron may use this presynaptic control point to adjust efficacy of synaptic transmission.

Quantal size can be altered by vesicular neurotransmitter transporter expression on vesicles

At excitatory synapses, observations of the effects of vesicular glutamate transporter (VGLUT) expression on synapse physiology have revealed that vesicular neurotransmitter transporters play important roles in regulating communication. I survey the glutamatergic literature as an example of how VMAT play a similarly important role in regulating

monoaminergic synapse physiology. Mass spectrometry of a prototypic (averaged) SV in the rat brain estimates the baseline VGLUT composition at 25 VGLUTs per vesicle (TAKAMORI *et al.* 2006), but this averaged approach hides variations in stoichiometry that occur during development or in response to experience. Although one VGLUT functional unit may be sufficient to fill a SV (DANIELS *et al.* 2006), more VGLUTs may be present on the vesicular membrane to adjust the rate or extent of vesicular filling. As it happens, VGLUT upregulation during development and *in vitro* overexpression corresponds to increased amounts of glutamate per vesicle (WILSON *et al.* 2005; WOJCIK *et al.* 2004). Similarly, VGLUT knock-out mammals display decreased amounts of glutamate per vesicle (FREMEAU *et al.* 2004; WOJCIK *et al.* 2004). In *Drosophila*, larvae with VGLUT overexpression display increased quantal size accompanied by a decrease in the number of vesicles released (DANIELS *et al.* 2004).

At aminergic synapses, VMAT upregulation also results in build-up of neurotransmitter within a vesicle with physiological ramifications. Using carbon fiber amperometry, Pothos and colleagues (2000) observed increase in quantal size and frequency in ventral tegmental area neurons overexpressing VMAT2 and further showed with high performance liquid chromotagraphy (HPLC) an increase in intracellular and released dopamine. As vesicles amass VMAT on its membranes, transmitter accumulates in vesicles and is reflected as a larger quantal size. They further postulate that VMAT recruited to vesicles which had not previously expressed VMAT fills empty vesicles and is reflected as a higher frequency of release. Since postsynaptic receptors are not saturated at all synapses (FRERKING and WILSON 1996; ISHIKAWA *et al.* 2002), differences in neurotransmitter accumulation within a vesicle and differences in the number of filled vesicles will activate postsynaptic targets to different degrees.

A pivotal role in regulation of aminergic neurotramission: Trafficking motifs which target VMAT to synaptic vesicles

Trafficking motifs target VMAT to the nerve terminal and to synaptic vesicles. Chapter 2 examines putative C-terminus motifs identified *in vitro* to traffic VMAT to synaptic vesicles. Using real-time imaging of VMAT tagged with a pH-sensitive fluorescent marker (pHluorin), I track VMAT trafficking during rounds of exo- and endocytosis. The pHluorin tag is further exploited to distinguish VMAT residing in sequestered acidic subcellular compartments. I present data to show that the previously identified YXXØ motif and additional motifs in the C-terminus of *Drosophila* VMAT not only regulate localization to synaptic vesicles and efficient endocytosis, but also trafficking to axonal terminals. My colleagues and I then show that these trafficking mutants show deficits in select behaviors, suggesting that some behaviors may not require tightly regulated synaptic transmission but rather communicate through volume transmission.

Complex relationship between central aminergic circuits

In mammals, a remarkable degree of crosstalk between aminergic systems has been observed. Studies of amphetamine-induced locomotor activity have suggested that serotonin suppresses catecholamine-induced behavioral arousal (GAINETDINOV *et al.* 1999; MABRY and CAMPBELL 1973). In addition, noradrenergic inputs regulate dopaminergic activity. Norepinephrine reduces the firing activity of dopaminergic neurons, and adrenergic antagonists and agonists modulate the strength of this interaction. Desipramine, a norepinephrine reuptake inhibitor, prolongs this effect, and idazoxan, an a2-adrenoceptor antagonist dampens this effect (GUIARD *et al.* 2008). Crosstalk between the serotonergic and dopaminergic system has also

been observed. In 5-HT$_{2C}$Rs mutant mice, the nigrostriatal dopaminergic system is hyperactive and behavior is responsive to treatment with a dopamine receptor agonist (ABDALLAH *et al.* 2009). These studies suggest that a remarkable level of interdependence and coordination exists between aminergic circuits.

The experiments in Chapter 3 exploit the genetic capabilities in *Dorosphila* to supplant individual amine circuits in an amine-silenced background in order to examine cooperativity or redundancy built into aminergic circuits.

Building aminergic circuits: Cooperativity or redundancy?

Previous studies examining the role of aminergic pathways in various behaviors have used cell-specific drivers to drive toxins or mutations affecting synaptic transmission (FRIGGI-GRELIN *et al.* 2003; KITAMOTO 2002; SUSTER and BATE 2002). Experiments which targeted expression of tetanus toxin in a subtype of aminergic neurons have suggested that dopamine may function as an inhibitor of startle-induced excitability and that tyramine and octopamine may have opposing effects on activity at the neuromuscular junction (FOX *et al.* 2006; FRIGGI-GRELIN *et al.* 2003). Although genetic ablation studies provide information on whether a specific cell type is necessary in a neuronal network, they do not provide information on whether a particular cell type is sufficient. Ablation studies where only one amine system is perturbed at a time are unable to unmask any redundant role between amine systems. Therefore, a rescue strategy which incrementally supplants amine release in an otherwise amine-silenced background provides a useful role in understanding individual amine contributions. The approach taken in Chapter 3 utilizes the normal mechanisms of vesicular neurotransmitter release, mimicking

endogenous patterns of neurotransmitter release and preserving amine circuitry to examine whether amine interact cooperatively or redundantly in circuits.

Defining temporal requirement for vesicular neurotransmitter transporters

Less work has been done to genetically dissect the temporal components of neuronal networks. Developmental deficits may be solely responsible for adult mutant phenotypes, but studies in mammals have been limited, likely because they are technically difficult. Some studies have shown that early-life blockade of serotonin reuptake transporters, either pharmacologically with selective serotonin reuptake inhibitors or genetically, alters emotional behavior in adult mice (ANSORGE *et al.* 2008; ANSORGE *et al.* 2004). It is likely that developmental deficits are also responsible for the adult mutant phenotype in VMAT2 knockout mice. In Chapter 4, I exploit the genetic tools available in *Drosophila* to examine the temporal requirement for two vesicular transmitter transporters: VMAT and vesicular GABA transporter (VGAT).

CHAPTER TWO:

Probing synaptic vesicle dynamics in a *Drosophila* vesicular monoamine transporter (dVMAT) trafficking mutant

SUMMARY

Membrane trafficking regulates the function of many synaptic proteins in vitro, but its effects on synaptic transmission and behavior remain unclear. The Drosophila vesicular monoamine transporter (DVMAT) regulates the loading and storage of monoaminergic transmitters in secretory vesicles, and proper localization to vesicles is required for the exocytotic release of neurotransmitters such as dopamine and serotonin. To test the potential effects of altered DVMAT trafficking on amine-dependent behaviors, I compared the ability of wild type and trafficking mutant transgenes to rescue a mutation in the endogenous DVMAT gene. Gradient fractionation experiments show that a specific DVMAT C-terminal mutation that removes two critical trafficking motifs fails to traffic DVMAT properly to secretory vesicles. My studies use pH-sensitive pHlourins to examine the vesicle dynamics during exocytosis and endocytosis in Drosophila expressing either the wild-type or mutant DVMAT. My findings suggest that the C-terminus contains trafficking motifs that slow endocytosis kinetics and dampen the presynaptic neuron's ability to recruit vesicles to the plasma membrane. Moreover, the previously described Δ3 deletion disrupts VMAT trafficking to the axon terminal. Combined with results showing that behaviors are differentially sensitive to mutations, these data are among the first to demonstrate that mislocalization of a synaptic protein may preferentially affect specific neuronal circuits and argue that behaviors require varying stringency levels for regulated neurotransmitter release.

INTRODUCTION

Neurotransmitter release requires vesicular neurotransmitter transporter localization on secretory vesicles. The extent of localization impacts vesicle filling and consequently communication strength to the postsynaptic target (POTHOS *et al.* 2000; WILSON *et al.* 2005). In amingeric neurons, vesicular monamine transporters (VMATs) package dopamine, serotonin and other amines into synaptic vesicles and large dense cores vesicles and localization to vesicles can determine the extent of neurotransmitter filling. In PC12 cells and transfected hippocampal neurons, a dileucine (Ile-Leu) motif in the C-terminus of the neural isoform of mammalian VMAT has been shown to be necessary for endocytosis (LI *et al.* 2005; TAN *et al.* 1998). Despite the progress made to identify trafficking motifs in cultured dissociated neurons, only recently have researchers examined endocytosis motifs in neurons *in vivo*. *In vivo* studies have found that a tyrosine-based endocytosis motif (YXXØ) directs dVMAT endocytosis and localization to synaptic vesicles (GRYGORUK *et al.* 2010b).

During synaptic vesicle biogenesis, proteins bound for synaptic vesicles at the nerve terminal are thought to first traffic to the plasma membrane (HANNAH *et al.* 1999; REGNIER-VIGOUROUX *et al.* 1991) and vesicular monoamine transporters are likely to follow a similar pathway (PRADO and PRADO 2002). Synaptic proteins exit the *trans* Golgi network (TGN) located in the somata and presumably traffic to axon terminals and other release sites via anonymous constitutive secretory vesicles (REGNIER-VIGOUROUX *et al.* 1991). It remains possible that some synaptic proteins exit the TGN on more special organelles, but it is generally accepted that vesicles exiting the TGN do not contain the complete complement of proteins found on mature synaptic vesicles and require additional trafficking steps to fully mature

(HANNAH *et al.* 1999) either at the nerve terminal (HANNAH *et al.* 1999; NAKATA *et al.* 1998) or at the soma (SANTOS *et al.* 2001). Studies have yet to examine the signals involved with directing vesicles to release sites and the signals involved with synaptic vesicle maturation in neurons *in vivo.*

In addition, mechanisms regulating VMAT localization to synaptic vesicle (SV) may differ from the signals used to direct the transporter to large dense core vesicle (LDCV), since biogenesis occur through separate routes (ARVAN and CASTLE 1998; REGNIER-VIGOUROUX *et al.* 1991). Unlike synaptic vesicles, LDCVs do not require an endocytosis step for their biogenesis (KELLY 1993). Instead, LDCVs and the proteins that reside on them are sorted into the regulated secretory pathway as they exit from the TGN (TOOZE and HUTTNER 1990). Several signals for sorting of rat VMAT2 to LDCVs have been identified *in vitro* and include acidic residues upstream as well as downstream of the dileucine motif (KRANTZ *et al.* 2000; WAITES *et al.* 2001). Specifically, the upstream glutamate residues are required for sorting into the regulated secretory pathway and onto immature LDCVs (KRANTZ *et al.* 2000; LI *et al.* 2005), whereas a phosphorylated acidic cluster at the extreme C-terminus of VMAT2 helps retain VMAT2 in the LDCV as it matures (WAITES *et al.* 2001).

In *in vivo* studies, biochemical fractionation of cellular compartments found that Y600 is a major signal in the C-terminus of DVMAT for localizing the transporter to synaptic vesicles and that a larger deletion (Δ3) of the C-terminus did not decrease the localization to synaptic vesicles any further (GRYGORUK *et al.* 2010b). The classical YXXØ motif was first identified as a trafficking motif in mannose-6-phosphate receptor (MARKS *et al.* 1997; TROWBRIDGE *et al.* 1993). Further studies have shown that the mu subunits of plasma membrane adaptor (AP2)

complexes bind directly to the tyrosine-based signals of sorted proteins (MARKS *et al.* 1997; OHNO *et al.* 1995).

More recently, Grygoruk separated synaptic vesicles from large dense core vesicles using sucrose density gradient fractionation and found that the Y600A mutation in dVMAT shunts a disproportionate percentage of the transporter to large dense core vesicles. In contrast, Δ3 DVMAT, which lacks additional trafficking motifs, has a broad sedimentation pattern, suggesting that most of the truncated protein is neither on SVs nor LDCVs. Using markers for early endosomes, late endosomes, recycling endosomes, and lysosomes, Grygoruk characterized the resolution power provided by sucrose density fractionation to distinguish subcellular compartments. Her data suggest that Δ3 DVMAT may be inappropriately retained in late endosomes or lysosomes.

In this chapter, I explore the role of the tyrosine-based motif and other motifs found in the C-terminus using real-time, *in vivo* imaging. Recently developed pH-sensitive fluorescent proteins allow measurements of exocytosis and endocytosis kinetics by reporting vesicle lumen pH (Figure 2-1). After fusion to the plasma membrane, the pHluorin tag detects the decreased acidity of the extracellular fluid (pH 7.4) vesicle compared to the lumen interior (pH 5.6) and unquenches fluorescence. During endocytosis, the vesicle is reacidified and pHluorin is requenched. The kinetics of fluorescence reflects the time course of endocytosis (MIESENBOCK *et al.* 1998; REIFF *et al.* 2005). Corroborating with Grygoruk's data, my data show that elimination of a second motif contained with the DVMAT C-terminus allows amine storage but further reduces sorting to SVs and may block all regulated release from both SVs and large dense core vesicles. Moreover, additional motifs in the C-teriminus may be involved with trafficking DVMAT to axon terminals and other points of transmitter release.

RESULTS

Deletion mutants of DVMAT-A show exocytosis deficits in vivo

To capture real-time dynamics of DVMAT trafficking in intact circuits, my colleagues and I generated transgenic flies expressing wild-type (wt) and mutant pHlourin-tagged dVMAT. Grygoruk et al. (2010b) previously identified a tyrosine-based motif (YXXØ, where Ø represents a building hydrophobic residue) acting as a signal for DVMAT-A localization to synaptic vesicles (SVs) *in vivo*. Using site-directed mutagenesis, the tyrosine residue in this trafficking motif was mutated to an alanine and the cDNA of ecliptic pHluorin was inserted into the first luminal loop of DVMAT-A. In addition, to determine whether other residues may be important for DVMAT trafficking to synaptic vesicles (SVs), we generated transgenic lines expressing the Δ3 deletion. The Δ3 deletion removes a putative di-leucine motif ($L^{589}I^{590}$), which may be involved in endocytosis and sorting to synaptic vesicles, the YXXØ motif, and a downstream acidic patch (EEDE) (Figure 2-2). Using the well-characterized GAL4/UAS system I expressed DVMAT pHluorin in all neurons including glutamatergic neurons that make up the Type I NMJ and octopaminergic neurons that make up the Type II NMJ in the fly. Pan-neuronal expression of DVMAT pHluorins does not affect larval or adult viability, and the VMAT-pHluorins traffic to NMJs similarly to the endogenous transporter.

Imaging analysis focused on Type I neuromuscular junctions (NMJs) in larval fillet preparations. Axons innervating the NMJ were stimulated with a train of pulses (80 action potentials at 40Hz), and axon terminals at 1b boutons were imaged for changes in fluorescence intensity. Wild-type pHluorin-tagged DVMAT shows a robust increase in fluorescence (dF/F 21.1% ± 2.9, n=18) following stimulation of the neuron to allow exocytosis, which then decays

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as it undergoes endocytosis (Figure 2-3 A). In contrast, transgenic flies expressing Y600A

DVMAT and Δ3 DVMAT show a smaller increase in fluorescence (Figure 2-3 B-D, dF/F 15.6%

± 2.6, n=11 for Y600A and dF/F 9.5% ± 1.7, n=15 for Δ3 DVMAT), but only Δ3 DVMAT

display an exocytotic defect that is different than wt (one-way ANOVA, Bonferroni post-test,

p<0.05). Reduced synaptic vesicle localization will be reflected as reduced fluorescence

intensities during exocytosis, since fewer transporters are localized on vesicles that are recruited

to the plasma membrane during evoked release. My data suggests that disruption of the Δ3

region in dVMAT reduces transporter expression on synaptic vesicles and that YXXØ and other

motifs in the C-terminus are involved with the transporter's localization to synaptic vesicles

recruited during evoked release.

The C Terminus of DVMAT-A contains motifs necessary for efficient endocytosis in vivo

To quantify potential differences in the kinetics of endocytosis, data collected during the

first 30 seconds after stimulus offset were fit to a single exponential curve and decay constants

(reported as tau values) were compared between genotypes. Within the first four seconds of

stimulus offset, the majority of wt and Y600A VMAT are internalized (τ 4.4 sec ± 0.9 and 4.2

sec ± 0.8, respectively; See Figure 2-3 D). The Δ3 mutation slows endocytosis kinetics (τ 8.7

sec ± 3.4) compared to wt (Figure 2-3 D, one-way ANOVA, Bonferroni post-test, p<0.05).

Consistent with *in vitro* studies (GRYGORUK *et al.* 2010a), endocytosis is complete in DVMAT-A

deletion mutants but motifs in the C-terminus are responsible for the rate of endocytosis. Thus,

trafficking motifs appear to play a role in the efficiency of endocytosis.

YXXØ and additional C-terminus motifs involved in trafficking to axon terminal

It is possible that Y600A and Δ3 DVMAT are trafficked to synaptic vesicles but reside on SVs that are not release competent. In this case, fluorescence in response to electrical stimuli would appear diminished in mutants, because DVMAT are localized to a pool of vesicles that do not fuse to the plasma membrane and the pHluorin tag remains quenched in an acidic compartment. To examine if the axon terminal contains a pool of vesicles that is not release-competent, the axon terminal was bathed with a membrane-permeable alkaline, ammonium chloride (NH_4Cl), to reveal total dVMAT expression at the nerve terminal. NH_4Cl permeates all subcellular compartments and raises luminal pH, so all pHluorin tags are unquenched. Importantly, the pH of the ammonium chloride solution was matched to the baseline saline solution pH so that changes in fluorescence reflect a change in luminal pH rather than a change in overall pH levels. Interestingly, bath application of NH_4Cl reveals that Y600A and Δ3 VMAT have reduced expression at the nerve terminal (Figure 2-4 B) despite having matched expression levels under normal saline (Figure 2-4 A). These findings suggest that YXXØ and other C-terminus trafficking motifs may be involved not only in localization to synaptic vesicles and endocytosis efficiency, but also in trafficking DVMAT to axon terminals.

Δ3 VMAT is inappropriately retained at the cell body

The finding that deletion of C-terminus motifs may impair DVMAT transport the axon terminal raises the question of whether DVMAT is improperly retained at the cell body. The cell bodies of motoneurons which compose the neuromuscular junction are clustered in the ventral ganglion. To quantify the expression of DVMAT at cell bodies, ventral ganglia were dissected from flies expressing pHluorin-tagged full-length VMAT, Y600A VMAT and Δ3 VMAT and ventral ganglion homogenates were probed on western blots using a GFP antibody that

recognizes the pHluorin tag. These results show that Δ3 VMAT is expressed more strongly at the ventral ganglion than Y600A or full-length VMAT (Figure 2-4 C). These results are consistent with epifluorescent images taken of intact ventral ganglia and neuromuscular junctions which show Δ3 VMAT expressed more strongly than wt at the ventral ganglion despite having equal or less than equal expression at the nerve terminal (Figure 2-4 D).

DISCUSSION

Prior to monominergic transmitter release, vesicles biogenesis must occur, vesicles must be transported to release sites and either large dense core vesicles or synaptic vesicles must be recruited for exocytosis. The vesicular monoamine transporter (VMAT) plays a critical role in this sequence of events by readying vesicles for neurotransmitter release competence. Several trafficking motifs in the VMAT C-terminus have been identified which direct the transporter for endocytosis and to synaptic vesicles. The experiments presented in this chapter tracked VMAT trafficking *in vivo* in whole intact circuits of various mutants. In order to uncover the role that the trafficking motifs play in directing VMAT for proper localization during the multi-step process, I examined VMAT recruitment to the plasma membrane during evoked release, localization at the axon terminal and retained expression at the cell body.

The experiments described here track pHluorin-tagged VMAT variants in a wild-type background which continues to express endogenous VMAT. The neuromuscular junctions observed continue to utilize synaptic vesicles which may express endogenous VMAT. In addition, transporter expression, whether tagged with pHluorin or not, is not expected to impact vesicle biogenesis. Vesicles will be present in the neuron regardless of whether the transporters

reside on vesicular membranes. Knockout mice which lack VMAT2 show no defects in synaptic

vesicle recycling (CROFT *et al.* 2005).

Here, I track VMAT by incorporating a pH-sensitive pHluorin to the first luminal loop of

the transporter. It has been reported that G-proteins bind to the first luminal loop of VMAT in

order to regulate transmitter uptake (BRUNK *et al.* 2006). However, it is unlikely that insertion of

a pHluorin tag on the luminal loop affects VMAT functionality because introduction of an HA

epitope tag does not change transport activity or VMAT localization in S2 cells (GREER *et al.*

2005; KRANTZ *et al.* 1997; WAITES *et al.* 2001).

Synaptic vesicles (SV) may be recruited from two possible pools: the exo/endo cycling

pool (ECP) and the reserve pool (RP). The ECP includes the functionally defined readily

releasable pool (RRP) and the immediately releasable pool (IRP) identified by Delgado and

colleagues and likely corresponds to the "docked" vesicles found juxtaposed to active zones

(KIDOKORO 2006). The reserve pool at the Drosophila NMJ is defined the same as in the

mammalian system, namely a large reservoir of synaptic vesicles that is released only during

intense stimulation (RIZZOLI and BETZ 2005). After ECP vesicles have been depleted of

neurotransmitters, SV are recruited from the RP for exocytosis, although empty vesicles continue

to be recruited from the ECP. Synaptic vesicles in the reserve pool are separated from the ECP

by actin during rest but are recruited during high-frequency stimulation by activating a cAMP-

dependent protein kinase A (PKA) pathway (KIDOKORO *et al.* 2004; KUROMI and KIDOKORO

2000). At the Calyx of Held, the readily releasable pool (RRP) has been further separated into

fast- and slow-releasing synaptic pools, termed RFP and SRP respectively (SAKABA and NEHER

2001). If ammonium chloride bath applications of fillet preps show that Y600A are trafficked to

the nerve terminal to some extent and are appropriately exported out of the cell body, then it is

possible that VMAT with the Y600A mutation are inappropriately retained in a SRP. Experiments at the Calyx of Held would predict that 30 action potentials delivered that 100 Hz would recruit synaptic vesicles from the SRP and any pHluorin-tagged VMAT residing on these vesicles would fluoresce with this high frequency stimulation but not under normal stimulation paradigms (LEE *et al.* 2012).

The results from the ammonium chloride bath application primarily reveal that C-terminus motifs are also involved with trafficking DVMAT to the axon terminal, but post-NH_4Cl pHluorin intensity differences between the $\Delta 3$ VMAT and Y600A VMAT also reveal that Y600A may sequester VMAT on synaptic vesicle pools or another compartment in the secretory pathway. Lumenal pH in organelles in the secretory and endocytic pathway are not uniform (CASEY *et al.* 2010; PAROUTIS *et al.* 2004), so pHluorin intensity changes in response to NH_4Cl application will differ depending on where aberrant VMAT is localized. As proteins move along the secretory pathway, the organelle lumen becomes progressively more acidic, with the endoplasmic reticulum luminal pH reported to be at 7.2, cis Golgi at 6.7 and trans Golgi at 6.0. There also appears to be a pH difference between acidic compartments in the constitutive pathway (pH 5.7) and regulated pathway (pH 5.2) prior to vesicle fusion to the plasma membrane. The change in pHluorin intensity elicited by electrical stimuli likely reflects movement from the acidic lumen of a synaptic vesicle in the regulated pathway (pH 5.2) to the more alkaline extracellular space; however, I cannot exclude the possibility that pHluorin-tagged VMAT may reside on other organelles in the secretory pathway and experience a different pH change. The indeterminate nature of the pH change also prevents calculation of the number of transporters or the number of vesicles which contain the misplaced DVMAT.

Examination of VMAT localization to synaptic vesicles has focused on axon terminals as the primarily location of vesicular release; however, vesicles also fuse to somatodendritic regions of the plasma membrane. Understanding of transporter and organelle traffic has largely overlooked differences between traffic to axons and dendrites (OVERLY *et al.* 1996). Studies examining regional differences in organelle motility suggest that endosomes and synaptic vesicle precursors exhibit net anterograde transport in axons but have no net movement in dendritic regions (OVERLY *et al.* 1996). These observations of differences between axonal and dendritic trafficking suggest that some trafficking machinery at the cell body must be in place to sort organelles. Differential guidance of vesicles to the axon versus dendrite are particularly relevant to trafficking of monoamine-containing vesicles since monoamines are packaged to both synaptic vesicles and large dense core vesicles and these vesicles differ in their subcellular sites of release (DE CAMILLI and JAHN 1990). Studies of trafficking to axon terminals and dendrites also need to consider mechanisms which anchor organelles to maintain proper organelle distribution (CHADA and HOLLENBECK 2004). My finding that Δ3 DVMAT is preferentially expressed in the cell body rather than at axon terminals may reflect either a defect in active transport to the axon terminal or retention defect where axon terminals are unable to dock vesicles containing DVMAT after successful traffic to the nerve terminal.

Precise neurotransmitter release is necessary for behavior. Phasic, unidirectional release of dopamine is detected by postsynaptic targets with tens of milliseconds precision and distinguishes neural encoding of reward from coding of aversive stimuli (SCHULTZ 2007). The pHluorin results show that the Δ3 deletion retards endocytosis rates and possibly fewer vesicles are recruited for neurotransmitter release with electrical stimulation. Synaptic vesicles which express Δ3 DVMAT likely release amine neurotransmitters in more graded, or ambient, manner

compared to wild-type. In our lab, Anna Grygoruk and Rod Najibi have shown that null

DVMAT mutants rescued with dVMAT carrying with the Δ3 deletion can selectively disrupt

some behaviors in the adult fly, e.g. those responsible for female sterility but not those required

for locomotion and escape responses. Their data therefore support the surprising idea that some

amine-dependent behaviors may depend only on ambient, steady state level of amines rather than

precisely timed synaptic release.

But, the idea of minimal requirements for amine release as ambient, steady state levels is

not new. Monoamine neurotransmission is thought to occur via two separate pathways: synaptic

(wiring) transmission and non-junctional, extrasynaptic (volume) transmission (AGNATI *et al.*

1995; FUXE *et al.* 2009). Synapse physiologists have observed more extrasynaptic release events

on axons, somata, and dendrites than had previously been thought (DE-MIGUEL and TRUETA

2005; DE WIT *et al.* 2009; HARTMANN *et al.* 2001; JAFFE *et al.* 1998). Monoamines, in particular,

likely modulate the neural circuitry – primarily composed of glutamatergic excitatory

connections and GABAergic inhibitory connections – by releasing into the extracellular space in

a diffuse manner (FUXE *et al.* 2007; FUXE *et al.* 2010; RICE and CRAGG 2008). Ambient, diffuse

neurotransmitter release may also impact postsynaptic targets very differently than targeted

synaptic release (FLORESCO *et al.* 2003).

Our findings begin to categorize behaviors as either requiring precisely timed synaptic

release or ambient, steady state release. Development of therapeutic drugs targeting the amine

system has considered mechanisms to handle the dynamic nature of neurotransmitter

dysregulation. As drug design consider factors such as feedback mechanisms involved with

regulation, phasic and tonic components of dysregulation, basal and activated release, and

compartmentalized release (SARTER *et al.* 2007), our findings are useful for identifying which

synapses require precise regulation to modify a behavioral output. Alternatively, drugs which mimic transmitter release, for example by blocking reuptake or acting as an agonist on postsynaptic receptors, do not yet reproduce the phasic pattern of neurotransmission (SARTER *et al.* 2009) and drug development may be more effective by targeting behaviors which have less stringent requirements for precisely timed neurotransmission.

FIGURES

Figure 2-1.   pH-sensitive pHluorins placed on luminal loop of vesicular protein fluoresces brighter when vesicles fuse to the plasma membrane.

pH-sensitive fluorescent tag (pHluorin) are quenched in acidic environments but fluoresce in alkaline environments. When pHluorin tag is placed on luminal loop of vesicular neurotransmitter transporter, the tag is exposed to the more alkaline extracellular space during exocytosis and fluoresces more brightly.
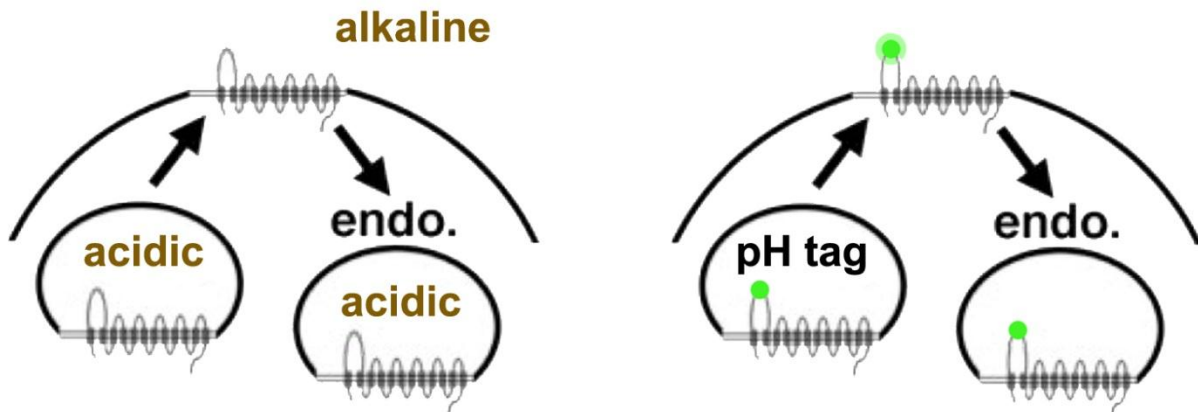
Figure 2-2. VMAT-pHluorin variants test trafficking motifs in C-terminus.

dVMAT contains 12 transmembrane domains with candidate sorting signals encoded in the C-terminus. Grygoruk et al. (2010) previously showed that Y600 (Y colored red in figure) is the first residue in a YXXØ motif, where the Y603 can be substituted by a bulky residue. Other candidate motifs include upstream and downstream acidic residues (colored blue in figure), which may be involved in maturation of large dense core vesicles (LV), and a LI motif, which may be required for endocytosis and sorting to synaptic vesicles (SV). The Δ3 deletion encodes a stop codon just upstream of the $L^{589}I^{590}$, thus removing the LI motif, YXXØ motif and downstream acidic patch (EEDE).
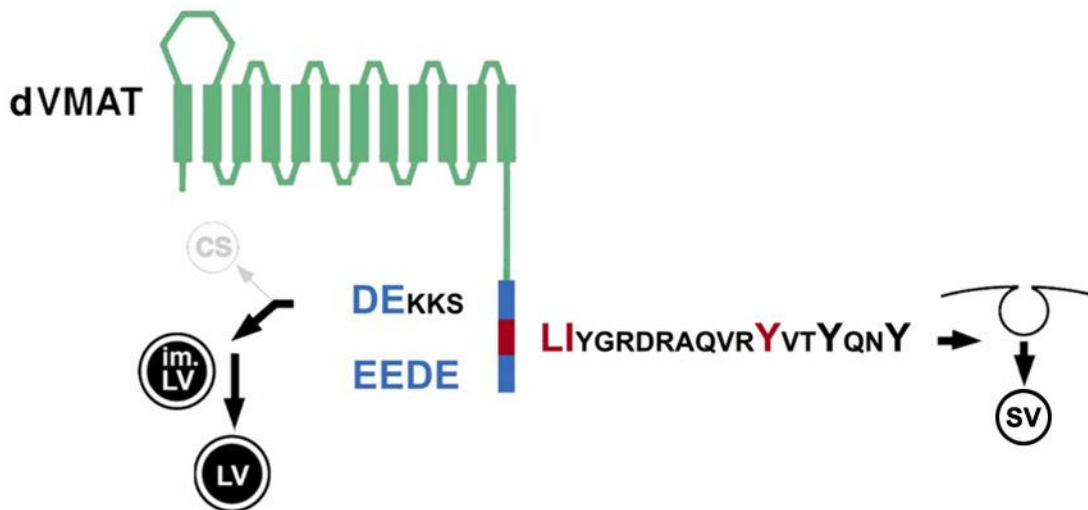
Figure 2-3. Trafficking motifs in dVMAT C-terminus direct transporter localization to vesicles and dictate endocytosis kinetics.

(A) A representative trace of pHluorin-tagged full-length dVMAT, showing fluorescence intensity changes in response to 80 action potentials (APs) at 40Hz. Boutons measured at muscle 13 in segment A4 in wild type (wt) animals expressing *UAS-full-length-dVMAT* under the control of a neuronal GAL4 driver (*e155—GAL4*). (B) A representative trace of pHluorin-tagged dVMAT with the Y600A mutation. (C) A representative trace of pHluorin tagged dVMAT with the Δ3 truncation, previously described in Grygoruk et al. (2010b). (D) Disruption of Δ3 region in dVMAT reduces transporter expression on synaptic vesicles. Reduced localization on synaptic vesicles is displayed as minimized fluorescence intensity during exocytosis. Peak fluorescence, reported as difference change relative to baseline fluorescence, in animals with pHluorin tagged full-length VMAT (n=18), Y600A mutant VMAT (n=11) and Δ3 mutant dVMAT (n=15). (E) Δ3 mutant dVMAT (n=14) endocytose at a slower rate than wt (n=16) or Y600A dVMAT (n=8). Gray bars indicate timing and duration of electrical stimulation.
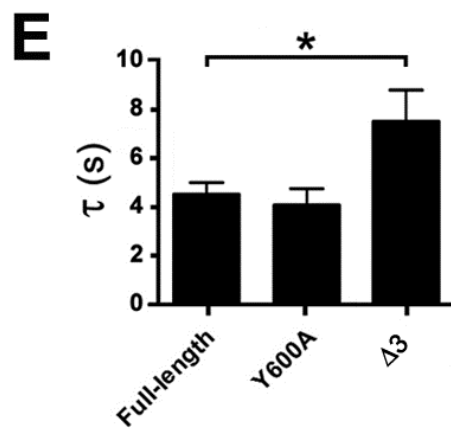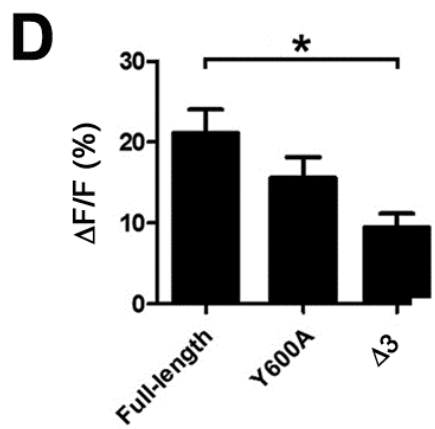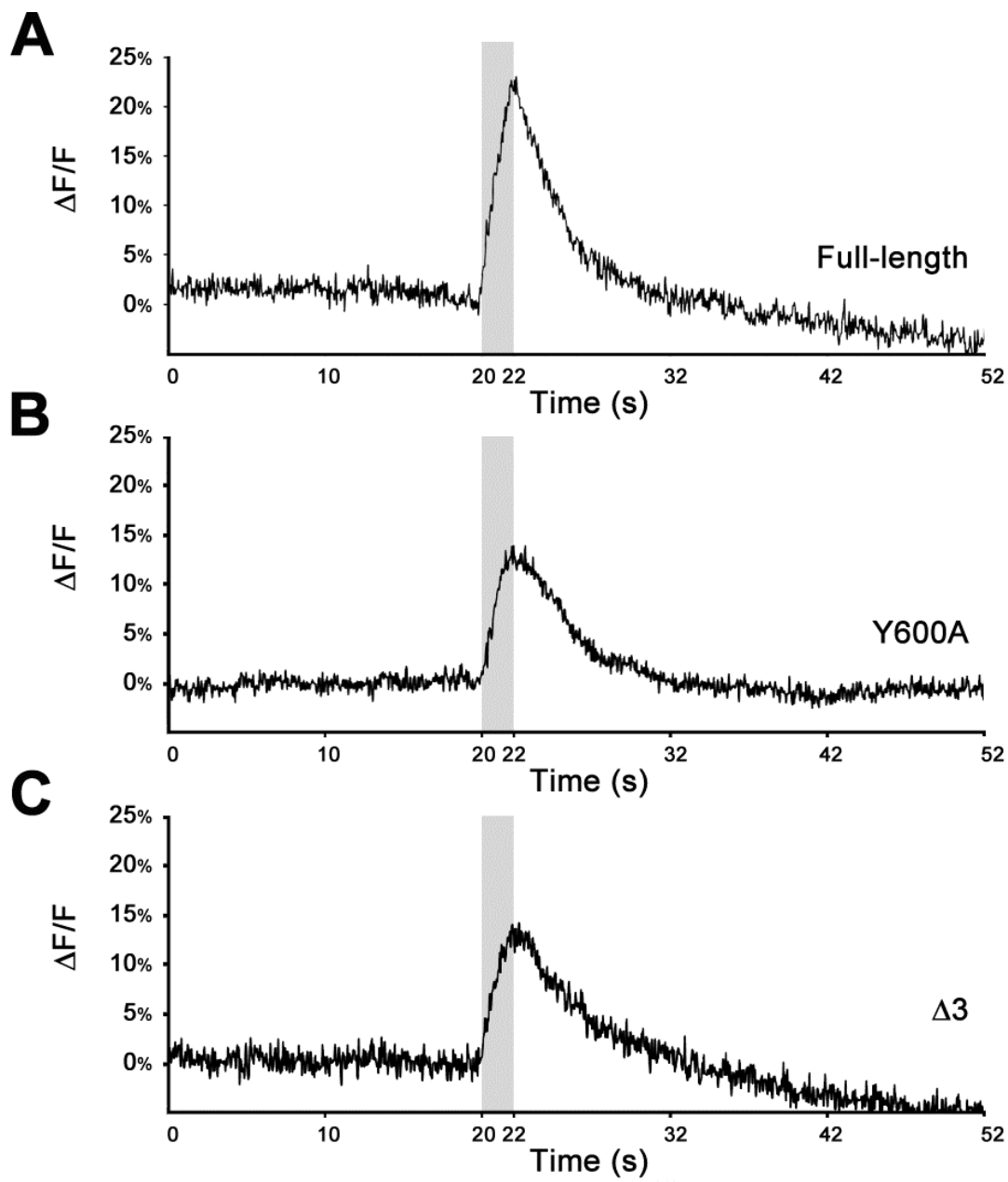
Figure 2-4. Δ3 region encodes motifs directing dVMAT to axon terminal.

(A-B) Application of a membrane-permeable alkaline reveals dVMAT-pHluorin normally

fluorescently quenched in acidic stores at the nerve terminal and reveals total dVMAT

expression at the nerve terminal. Bath application of an ammonium chloride solution reveals

that Y600A VMAT and Δ3 VMAT have reduced expression at the nerve terminal (B) despite

having similar expression levels under normal hemolymph-like saline (A).

(C-D) Δ3 VMAT is inappropriately retained at the ventral ganglion, where the cell bodies are

located. (C) Homogenates of the ventral ganglion from flies expressing pHluorin-tagged full-

length VMAT, Y600A VMAT and Δ3 VMAT were probed on western blots using a GFP

antibody that recognizes the pHluorin tag and a β-tubulin antibody that acts as a loading control.

Δ3 VMAT is expressed more strongly at the ventral ganglion than Y600A or full-length VMAT.

(D) Epifluorescent images showing Δ3 VMAT expressed more strongly than full-length VMAT

at the ventral ganglion, but more weakly at the nerve terminal. Images were taken at baseline

under normal hemolymph-like saline (pH 7.32).
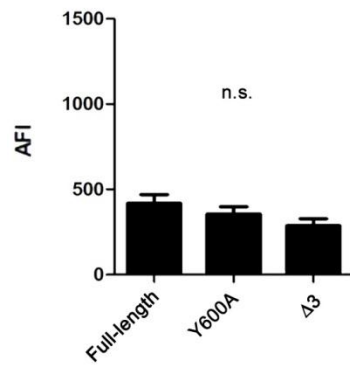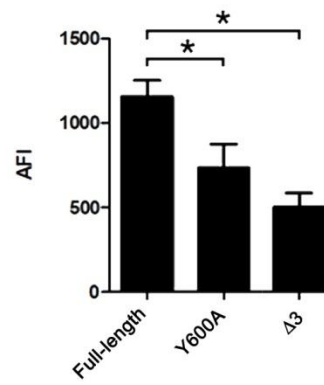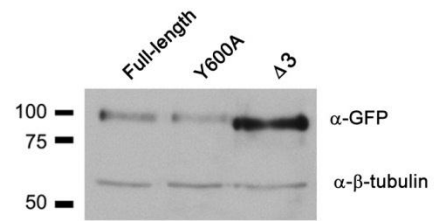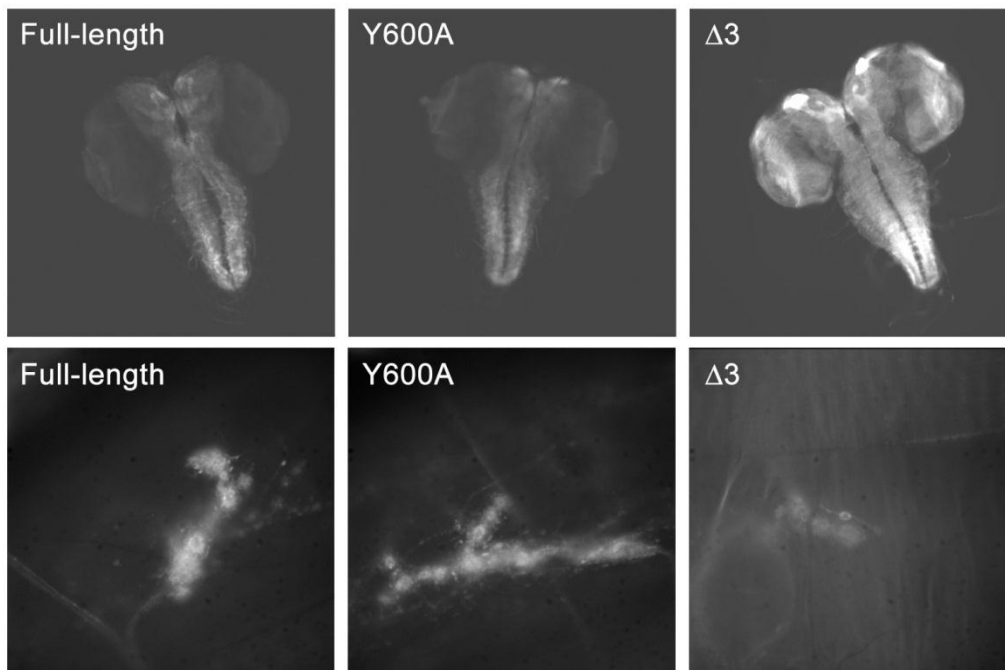
CHAPTER THREE:

Selective neuronal restoration of vesicular monoamine transporter function uncovers coordinated, complementary and dispensable roles for dopamine, octopamine and serotonin

SUMMARY

To investigate the regulation of *Drosophila melanogaster* behavior by biogenic amines, I exploited the broad requirement of the vesicular monoamine transporter (VMAT) for all aminergic synaptic transmission. I used the Drosophila *VMAT (dVMAT)* null mutant to globally ablate neuronal amine release, and then restored DVMAT activity in either individual or multiple aminergic systems using transgenic rescue techniques. I find that larval survival, larval locomotion, and female fertility rely predominantly on octopaminergic circuits with little apparent input from either serotonin or dopamine. In contrast, male courtship and fertility can be rescued by expressing DVMAT in octopaminergic or dopaminergic neurons, suggesting potentially redundant circuits. Rescue of major aspects of adult locomotion and startle behavior required octopamine, but complementary roles were observed for serotonin and dopamine. Interestingly, adult circadian behavior could not be rescued by expression of DVMAT in a single subtype of aminergic neurons, but required at least two systems, suggesting the possibility of unexpected cooperative interactions. These data provide a template for possible modes of regulation by monoamines that may help define the mechanism of action of aminergic circuits in Drosophila as well as other systems.

INTRODUCTION

Both invertebrate and mammalian behaviors undergo extensive regulation by monoamine neurotransmitters (Reviewed in BARRON *et al.* 2010; BROMBERG-MARTIN *et al.* 2010; HAENISCH and BÖNISCH 2011; JOSHUA *et al.* 2009; SARA 2009; SCHULTZ 2010; SHOHAMY and ADCOCK 2010). These include serotonin (5-HT), dopamine (DA), noradrenaline (NE), adrenaline and histamine in mammals (BARNES *et al.* 2011; DAUBERT and CONDRON 2010; HAENISCH and BÖNISCH 2011; SARA 2009; SCHULTZ 2010; SHOHAMY and ADCOCK 2010; SOUTHWICK *et al.* 2005), and 5-HT, DA, histamine, octopamine (OA) and tyramine (TA) in Drosophila as well as other insects (BARRON *et al.* 2010; BLENAU and BAUMANN 2001; COLE *et al.* 2005; DAUBERT and CONDRON 2010; FAROOQUI 2007; HARDIE *et al.* 2007; MONASTIRIOTI *et al.* 1996; ROEDER 2005; WADDELL 2010). The function of individual aminergic systems has been studied with both genetic and pharmacologic methods, yet the contribution of each system and the manner in which they may interact to regulate complex behaviors remains unclear. Understanding these interactions will be critical to determine not only the molecular mechanisms by which amines regulate behavior, but also the contribution of each system to the therapeutic effects of antidepressants, antipsychotics and other psychotropic drugs (HAENISCH and BÖNISCH 2011; MAILMAN and MURTHY 2010; MELTZER *et al.* 2008; SORA *et al.* 2001; WILENS 2006).

Studies in mammals illustrate the complex relationship between central aminergic circuits (GUIARD *et al.* 2008; LEGGIO *et al.* 2009; MABRY and CAMPBELL 1973; SESACK and GRACE 2010). These include the regulation of DA release by 5-HT (ABDALLAH *et al.* 2009; ALEX and PEHEK 2007; DI MATTEO *et al.* 2001), as well as the convergence of 5-HT and DA onto some of the same downstream outputs (BOUREAU and DAYAN 2010; GERVAIS *et al.* 1999). However,

studies of possible interactions between aminergic systems in mammals are limited by technical

constraints usually associated with combining multiple knockout alleles. Most behavioral-genetic

models therefore study the effects of inhibiting one, or at most, two aminergic systems. This

approach can limit the analysis of potential interactions: since each of these regulatory systems

may be either redundant or replaceable by other aminergic systems, knocking out one component

may not alter behavioral phenotypes of interest. For example, the combined contributions of 5-

HT, noradrenaline, and DA to the behavioral effects of cocaine remained unclear until double

and triple knockouts of the 5-HT, DA and noradrenaline transporter genes were generated

(MÖSSNER *et al.* 2006).

Behavior in model systems such as *C. elegans* (CHASE and KOELLE 2007; DUERR *et al.*

1999; SANYAL *et al.* 2004; SAWIN *et al.* 2000; SUO *et al.* 2009) and Drosophila are also

dependent on multiple aminergic regulatory systems (BARRON *et al.* 2010; BLENAU and

BAUMANN 2001; COLE *et al.* 2005; DAUBERT and CONDRON 2010; FAROOQUI 2007; HARDIE *et al.* 2007; MONASTIRIOTI *et al.* 1996; ROEDER 2005; WADDELL 2010). In Drosophila, molecular-

genetic and pharmacologic analyses have demonstrated the importance of DA in sleep, arousal

and aversive conditioning (ANDRETIC *et al.* 2005; BAYERSDORFER *et al.* 2010; CHANG *et al.*

2006; DRAPER *et al.* 2007; KUME *et al.* 2005; NECKAMEYER and WEINSTEIN 2005; SCHWAERZEL

et al. 2003), 5-HT in aggression, place memory, circadian rhythms, and sleep (ALEKSEYENKO *et al.* 2010; SITARAMAN *et al.* 2008; YUAN *et al.* 2006; YUAN *et al.* 2005), and OA in sleep,

locomotion, female fertility, aggression, place memory, and the fly's response to environmental

stressors (CROCKER and SEHGAL 2008; CROCKER *et al.* 2010; FOX *et al.* 2006; HARDIE *et al.*

2007; HIRASHIMA *et al.* 2000; LEE *et al.* 2003; SITARAMAN *et al.* 2010; ZHOU *et al.* 2008).

However, as in mammals, the relative importance of each aminergic system for particular behaviors and their potential interplay remains unclear.

To explore the function and interaction of aminergic systems in Drosophila, I have taken advantage of the requirement for the vesicular monoamine transporter (VMAT) in the vesicular storage and exocytotic release of all biogenic amines within independent subsets of aminergic neurons (DUERR *et al.* 1999; GREER *et al.* 2005; LIU and EDWARDS 1997). My colleagues in the Krantz group have previously shown that flies express a single VMAT gene (*dVMAT*) in 5-HT, DA and OA neurons and that mutation of *dVMAT* causes multiple behavioral deficits (SIMON *et al.* 2009). The requirement of DVMAT for normal exocytotic transmitter release in all central aminergic neurons-- rather than a single subset-- presents a unique opportunity for ablating normal synaptic transmission all aminergic systems simultaneously then adding back each one in isolation or in combination. Thus, starting with a loss of function *dVMAT* mutant background, I have systematically added back, or genetically rescued, DVMAT function in specific subsets of aminergic neurons.

My approach differs from previous studies using single mutations, as well as those in which toxins or dominant transgenes have been used to silence individual transmitter systems or circuits, and circumvents some important limitations. First, ablating the function of one modulatory system will not reveal deficits if redundant or complementary regulatory circuits compensate for the loss. Second, although genetic ablation studies can determine how loss of particular systems or circuits will affect behavior, they do not describe the minimal circuitry that is both necessary and sufficient to drive any given behavior. Conversely, this approach allows us to determine whether a single system is sufficient to drive a particular behavior, since we start from a baseline of no exocytotic release in any aminergic system and then add back individual

amines. Moreover, unlike some other probes of aminergic processes (NECKAMEYER and QUINN 1989), *dVMAT* is not expressed in non-neuronal tissues such as those required for cuticle hardening and pigmentation (SIMON *et al.* 2009). This restricted pattern of expression renders DVMAT particularly useful for studying neuronal processes in the absence of potentially confounding non-neuronal effects (COLAS *et al.* 1999; HSOUNA *et al.* 2007; TRUE 2003). Finally, the mutation of *dVMAT* rather than biosynthetic enzymes also confines the signaling deficit to normal exocytotic release as opposed to other types such as efflux, the function of which is poorly understood.

My data indicate that restoration of DVMAT in cells that release OA and/or TA is sufficient to rescue larval locomotion, female fertility, and the decreased viability of *dVMAT* mutants. The findings are consistent with previous studies demonstrating the widespread importance of OA in multiple aspects of invertebrate behavior (BICKER 1999; CHASE and KOELLE 2007; ROEDER 1999; SCHEINER *et al.* 2006; VERLINDEN *et al.* 2010). Both DA and OA can individually rescue male courtship and male fertility, suggesting complementary and potentially redundant roles for these systems in stimulating male sexual behavior. Individual aminergic systems are also able to partially rescue some of the defects seen in the *dVMAT* mutant startle response. In contrast, only combinations of aminergic systems are able to detectably rescue the defects we observe in circadian behavior. These differences may be generally relevant to the mechanisms by which aminergic systems interact to modulate behavior and respond to psychotropic drugs.

RESULTS

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Rescue Strategy

To determine which aminergic system(s) are required for survival under normal culture conditions, I compared the survival of *dVMAT* mutants rescued with each aminergic driver. Drivers used here included *Tyrosine decarboxylase-Gal4* (*Tdc2-Gal4*) to express *UAS-DVMAT* in octopaminergic and tyraminergic neurons, *Tyrosine Hydroxylase-Gal4* (*TH-Gal4*) to express *UAS-DVMAT* in dopaminergic cells, and *Tryptophan Hydroxylase-Gal4 (TrH-Gal4)* to express *UAS-DVMAT* in serotonergic cells. These drivers confer specificity by encoding promoter regions of enzymes used in the biosynthesis of a specific neurotransmitter (COLE *et al.* 2005; FRIGGI-GRELIN *et al.* 2003; ZHANG *et al.* 2004). Expression of DVMAT using each driver was confirmed on Western blots (Figure 3-1). As previously reported, we have also used the *daughterless-Gal4* (*da-Gal4*) driver to broadly express *UAS-DVMAT* throughout the fly and to rescue amines levels and the *dVMAT* mutant phenotype (SIMON *et al.* 2009). Since *UAS-DVMAT* driven by *da-Gal4* is not expressed in 5HT neurons, we designate rescue with *da-Gal4* in the experiments below as nominally ubiquitous with the notable absence of rescue in serotonergic neurons ("ubiq^{-5HT}").

To further demonstrate that expression using *TH-, TrH-* and *Tdc2-Gal4* conferred functional rescue, I worked with Niall Murphy and Rachel Kelly to perform HPLC with electrochemical detection (Figure 3-2) (CHANG *et al.* 2006). For measurement of DA and 5HT, homogenates were obtained from whole adult heads. Since the cuticle contains an unidentified compound with a column retention time similar to OA, we measured OA levels in homogenates of dissected adult brains (HARDIE and HIRSH 2006). Expression of *UAS-DVMAT* using the *Tyrosine Hydroxylase-Gal4* driver (Figure 3-2 A, *TH-Gal4)* (FRIGGI-GRELIN *et al.* 2003) restored DA to levels similar to wt flies (compare Figure 3-2 A and 3-2 B). We also see a trend toward

higher levels of DA using *Tryptophan Hydroxylase-Gal4* to express DVMAT (Figure 3-2 A).

Although this difference did not reach statistical significance, we cannot rule out the possibility

that the *Trh-Gal4* driver used in these studies is expressed at low levels in some dopaminergic

cells (PARK *et al.* 2006). Expression using the *Tryptophan Hydroxylase-Gal4* (Figure 3-2 C,

TrH-Gal4) (PARK *et al.* 2006) and *Tyrosine decarboxylase*2 (Figure 3-2 D, *Tdc2-Gal4)* (COLE *et*

al. 2005) partially rescued the tissue content of 5HT and OA respectively. Rescued levels of 5-

HT and OA were lower than wt (CS) flies (compare Fig. 3-2 C and D, 3-2 E and F) but appeared

to be more specific for the appropriate driver than rescue of DA using *TH-Gal4*.

Octopamine and tyramine are sufficient to rescue the density dependent survival deficit of the

dVMAT mutant

My colleagues have previously shown that the *dVMAT* mutation is lethal under standard

culture conditions but can survive under conditions of low density; only 5-10% of homozygous

dVMAT mutants reared under standard culture conditions survive to adulthood (SIMON *et al.*

2009). To determine whether this could be due to gross changes in neural development, Hakeem

Lawal and I performed an immunofluorescent analysis of larval aminergic neurons (Figure 3-3).

Of the seven DA clusters we analyzed, one (DL2) showed an ~17% decrease in total cell number

(Figure 3-3 A, B). The other DA clusters did not differ significantly from wt (Figure 3-3 A, B).

We did not detect any differences between wt and the mutant for any 5HT (Figure 3-3 C,D) or

OA cell clusters (Figure 3-3 E, F) in the larva. Since the DA, 5-HT and OA systems appeared to

be grossly intact, I proceeded with genetic experiments to rescue the storage and release of

individual amines using *UAS-DVMAT*; however, we cannot rule out the possibility that mutation

of *dVMAT* causes additional defects in the neuropil that could affect our analysis (see Discussion).

To determine which aminergic system(s) are required for survival under normal density culture conditions, we compared the survival of *dVMAT* mutants rescued with each aminergic driver (Figure 3-3 G and Figure 3-4). Expression of *UAS-DVMAT* using *TH-*, *TrH-* or *Ddc-Gal4* did not increase survival under standard (high density) culture conditions (Figure 3-3 G and Figure 3-4 A-C). In contrast, mutants rescued with either *Tdc2-* or *daughterless-Gal4* (Figure 3-3 G and Figure 3-4 D, E) survived at rates comparable to wild-type (wt) controls. Thus, although we cannot rule out additional effects of DA and 5-HT on development under low density conditions (see Figure 3-4), my data suggest that the exocytotic release of DA and 5-HT may not be required for viability under standard, high-density culture conditions. By contrast, OA and/or TA are sufficient to rescue the density-dependent lethality of the *dVMAT* mutant under standard conditions.

Octopamine is sufficient for the initiation of larval locomotion

dVMAT mutant larvae have grossly retarded locomotion (SIMON *et al.* 2009) consistent with other studies demonstrating aminergic regulation of crawling and fictive locomotion in intact larvae and *ex vivo* preparations respectively (FOX *et al.* 2006; NECKAMEYER 1996). 5-HT is important for regulation of locomotion in some invertebrates (MACKEY and CAREW 1983; NUSBAUM 1986; WILLARD 1981), and it has been previously suggested that both 5-HT and DA might contribute to the regulation of larval motor function in Drosophila (COOPER and NECKAMEYER 1999; MACKEY and CAREW 1983; NECKAMEYER 1996). Gabriel Seidman, a visiting undergraduate in the lab, and I do not detect a significant increase in locomotion in

larvae that express DVMAT in 5-HT neurons using *TrH-Gal4* (Figure 3-5 A, TrH), DA neurons using *TH-Gal4* (Figure 3-5 A, TH) or, in a separate set of experiments, both DA and 5-HT neurons using *Ddc-Gal4* (not shown). In contrast, DVMAT expression in OA+TA neurons using *Tdc2-Gal4* cells rescues the locomotor activity to levels approaching that of the wild type controls (Figure 3-5 A, Tdc).

Previous studies have shown that ingested amines can be taken up by cells in the central nervous system (BUDNIK *et al.* 1989) and thus rescue the behavior of mutants deficient in OA synthesis (MONASTIRIOTI *et al.* 1996). Similarly, 5-HT and DA feeding have been used to study their potential effects on ovarian follicle development (SEDORE WILLARD *et al.* 2006) and DA feeding has been shown to partially rescue the phenotype of the *Dopa Decarboxylase* (*Ddc*) mutant (BUDNIK *et al.* 1989). To explore the potential roles of particular amines in stimulating larval locomotion, Christine Djapri, a technician in the lab, fed homozygous *dVMAT* null mutants 10 mg/mL OA or other amines (Figure 3-5 B-C). *dVMAT* mutant larvae fed OA for either 2 or 4 hours showed enhanced locomotion relative to mutant controls (Figure 3-5 B). In contrast, feeding TA (Figure 3-5 C) had no effect on larval locomotion in the *dVMAT* mutant. Likewise, vehicle, DA, and 5-HT had no effect on larval locomotion in the *dVMAT* mutant (not shown). Although we cannot rule out the possibility that amines other than OA failed to rescue because these were absorbed at ineffective concentrations, these findings suggest that OA plays a dominant role in rescuing the larval locomotion defect seen in mutants.

Storage of octopamine by DVMAT is required for egg release

Female flies which lack DVMAT are infertile and retain more eggs in their ovaries than wt females (SIMON *et al.* 2009). Consistent with these earlier findings, I show that few *dVMAT*

mutants produce progeny (Figure 3-6 A, -/-). DVMAT expression in OA+TA+DA cells using

da-Gal4 rescues the fertility defect (Figure 3-6 A, ubiq^{-5HT}), and since *da-Gal4* does not appear

to drive expression in 5-HT neurons (SIMON *et al.* 2009) these data suggest that 5-HT is

dispensable for female fertility. Indeed, rescue of DVMAT expression in 5-HT cells using *TrH-*

Gal4 did not improve fertility of the *dVMAT* mutant (Figure 3-6 A, TrH). Rescue using *TH-Gal4*

alone was associated with a small, but statistically significant increase in fertility (Figure 3-6 A,

TH). In contrast, expression of DVMAT in TA+OA cells with *Tdc2-Gal4* restored female

fertility to nearly wild type levels (Figure 3-6 A, Tdc).

    To further explore the mechanism by which loss of OA storage and release reduces

fertility of the *dVMAT* mutant, I examined oocyte retention in the ovaries of mated female flies

(Figure 3-6 B, C). I find that expression of DVMAT in OA+TA cells (Tdc) rescues the oocyte

retention observed in the *dVMAT* mutants (Figure 3-6 B, C, Tdc). Hakeem Lawal, a postdoc in

the lab, also finds that feeding OA but not TA, or DA rescues the egg retention phenotype in the

dVMAT mutant (not shown). These results are consistent with previous reports showing that

mutation of the biosynthetic enzyme for OA causes egg retention (MONASTIRIOTI 2003) and that

OA receptors are likely to mediate passage of eggs through the oviduct as well (LEE *et al.* 2009;

LEE *et al.* 2003; MIDDLETON *et al.* 2006; RODRÍGUEZ-VALENTÍN *et al.* 2006).

    Although OA appeared to exert the most robust effects on female fertility and egg-laying,

we observe additional, possibly complementary effects of DA. Expression of DVMAT in DA

neurons alone modestly but significantly increased the fertility of the *dVMAT* mutant (Figure 3-6

A) and also showed a trend toward higher numbers of retained oocytes, although this did not

reach statistical significance (Figure 3-6 B, C, TH). These observations may reflect a role for

DA in oocyte development, independent of the role of OA in egg-laying (See Discussion).

Dopamine and octopmaine play complementary or redundant roles in male sexual behavior

We next used the *dVMAT* rescue lines to examine male sexual behavior (Figure 3-6 D-E). My colleagues have previously shown that male fertility is dramatically reduced in the *dVMAT* mutant (SIMON *et al.* 2009); however, the contribution of aminergic circuits to male courtship and mating remains poorly understood. I find that fertility of *dVMAT* mutant males is restored with the *da-Gal4* driver (Figure 3-6 D, ubiq^{-5HT}). In addition, both *TH-Gal4* and *Tdc2-Gal4* but not *TrH-Gal4* partially rescued male fertility (Figure 3-6 D).

My colleagues have previously shown that overexpression of DVMAT results in increased courtship activity (CHANG *et al.* 2006). My colleagues, Anna Grygoruk and Rod Najibi, show here that courtship is reduced in the *dVMAT* mutant (Figure 3-6 E, -/-). Courtship is partially rescued using either *Tdc2-Gal4* or *TH-Gal4* (Figure 3-6 E, Tdc and TH). Conversely, *TrH-Gal4* does not significantly rescue the male courtship defect observed in *dVMAT* mutant (Figure 3-6 E, TrH). Although neither *TH-Gal4* nor *Tdc2-Gal4* fully restores courtship to wild-type levels, these data suggest that both DA and OA contribute to important aspects of male sexual behavior. We note that although DA has been suggested to instigate same-sex courtship (LIU *et al.* 2009) and female receptivity (NECKAMEYER 1998b; WICKER-THOMAS and HAMANN 2008), neither DA nor OA have been clearly assigned a role in male-female courtship. Furthermore, since DA and OA can both rescue male fertility and courtship in the *dVMAT* mutants, we suggest that they serve complementary or perhaps redundant roles in male sexual behavior (See Discussion.)

The dVMAT mutant has altered startle-induced activity

Dopamine has been previously implicated in arousal and startle responsiveness in Drosophila (KUME *et al.* 2005; LEBESTKY *et al.* 2009). Although there are no reports on the role of other amines in Drosophila startle, OA has been suggested to increase arousal and mediate the fight-or-flight response in other insects (ADAMO *et al.* 1995; BACON *et al.* 1995; RIND *et al.* 2008; STERN 1999). In negative geotaxis assays, the diminished startle responsiveness in *dVMAT* mutants is measured as a lower percentage of flies that reach the top of a vial in response to mechanical startle (SIMON *et al.* 2009). Harshul Zaveri, an undergraduate, and I find that expression of DVMAT in OA+TA neurons via *Tdc2-Gal4* significantly improved performance in negative geotaxis assays whereas rescue with *TH-Gal4* or *TrH-Gal4* did not (Figure 3-7 A).

To further assess the role of OA and other amines in startle-induced locomotion, I worked with Tim Lebestky to utilize a repetitive startle assay (the puff-o-mat) that he designed to quantify parameters and phases within the startle response (LEBESTKY *et al.* 2009). In response to air puffs, wt controls increase locomotor speed within seconds and then gradually return to a less active state in a response profile that can be characterized by a single exponential (LEBESTKY *et al.* 2009). We find that *dVMAT* mutants show a dramatically reduced peak velocity relative to wt controls (Figure 3-7 B-E, -/-). Also consistent with earlier observations (SIMON *et al.* 2009), *dVMAT* mutants display a somewhat higher basal locomotion in the pre-startle phase, when acclimating to a novel environment, in comparison with wt animals (Figure 3-7 C, time 0 - 60 s, compare wt to -/-). This result suggests that although the mutants display an abrogated startle response, they are not generally sluggish in all locomotor measures.

Consistent with the results of negative geotaxis assays, we observe significant rescue of startle responsiveness in the puff-o-mat assays by restoring DVMAT expression in OA+TA

neurons (Figure 3-7 C). Further dissection of the startle response shows that selective restoration of DVMAT in OA+TA neurons restores peak response to startle (Figure 3-7 B) and distance travelled (Figure 3-7 F). However, *dVMAT* mutants rescued with *Tdc2-Gal4* continued to show heightened basal locomotor activity relative to wt controls (Figure 3-7 C, time 0-60s. Note that the peak velocity value reported in Figure 3-7 B is the difference between peak velocity and basal activity and corrects for differences in basal locomotor activity). Restoration of DVMAT expression using *Tdc2-Gal4* also resulted in a prolongation of the startle response, where the slow decay of the startle state is measured as a heightened tau value (Figure 3-7 C, G).

In contrast to the effects seen using the *Tdc2-Gal4* driver, restoration of DVMAT in DA (Figure 3-7 D) and 5-HT neurons (Figure 3-7 E) failed to rescue peak startle responsiveness (Figure 3-7 B) or distance traveled in the immediate, post-startle period (Figure 3-7 F). However, speed to reacclimation after startle, quantified as the rate constant tau, appeared to be rescued using either *TrH-Gal4* (Figure 3-7 G). In addition, asymptotic velocity of *dVMAT* flies recued with *TrH-Gal4* was significantly lower than of the *dVMAT* mutant and indistinguishable from wt (Figure 3-7 H). In sum, some aspects of adult locomotion and startle were rescued by expression in OA+TA cells, irrespective of the activity of other systems. In addition expression using *TrH-Gal4* showed effects on other aspects of the startle response, suggesting potentially complementary roles for each system in regulating adult locomotor and startle behaviors.

Circadian locomotor activity rhythms are abnormal in the dVMAT mutant

For both mammals and flies, the control of circadian rhythmicity is complex, and may involve multiple aminergic systems including 5-HT and DA (CROCKER *et al.* 2010; GRAVOTTA *et al.* 2011; HIRSH *et al.* 2010; KUME *et al.* 2005; MEIJER and GROOS 1988; MORIN and

BLANCHARD 1991; NÄSSEL and HOMBERG 2006; SUH and JACKSON 2007; YUAN *et al.* 2005).

However, it is unknown whether the different aminergic systems function independently or serve

redundant functions in the regulation of rhythmicity. Thus, I collaborated with Fanny Ng from

Tufts University to examine locomotor activity rhythms in *dVMAT* mutants and genetically

matched wild-type controls in both light:dark (LD) and free-running (constant dark or DD)

conditions using the Drosophila Activity Monitor (DAM) system. Fly activity was monitored for

3 days in a light:dark cycle consisting of 12 h of light and 12 h of dark (LD 12:12) and then

subsequently in DD conditions for an additional 7-8 days. As expected, Ng found that wt control

flies exhibited normal levels of locomotor activity and robust bimodal activity rhythms in LD

with peaks occurring at the beginning (morning) and end (evening) of day. This was observed in

actograms for individual flies and in plots illustrating average daily profiles of activity for an

entire population (not shown). Consequently, the average Rhythmicity Index (RI), a measure of

the robustness of the rhythm (LEVINE *et al.* 2002), was high for wild-type control populations. In

contrast, *dVMAT* mutants had reduced activity and weaker rhythmicity in LD (not shown). In

addition, the daily profile of activity was altered such that morning activity was proportionally

decreased relative to the evening bout of activity.

When transferred to DD, wild-type controls continued to show robust activity rhythms

(Figure 3-8 A) with a high average percent rhythmicity (Figure 3-8 B) and RI value (Figure 3-8

C). However, even for the *dVMAT* mutant population that exhibited bimodal rhythms in LD,

only ~40% had statistically significant rhythmicity in DD (Figure 3-8 A, B -/-). Thus, the

average RI value for the mutant population was significantly decreased relative to controls in

these conditions (Figure 3-8 C). Interestingly, the circadian molecular oscillator of neurons is

normal in the *dVMAT* mutant in both LD and DD conditions, as assessed by

immunohistochemical measurements of PERIOD (PER) abundance. Similarly, rhythms in

Pigment Dispersing Factor (PDF), a circadian neurotransmitter released from ventral lateral

clock neurons, appeared normal in the mutant. Both observations are consistent with the idea

that amines modulate circuitry downstream of the pacemaker.

We next tested whether expression of a *UAS-DVMAT* transgene in DA, OA+TA or 5-HT

cells could rescue rhythmicity of the *dVMAT* mutant. In contrast to other behaviors described in

this chapter, expression of DVMAT using any of the three individual aminergic drivers was not

able to rescue the activity rhythm defects of the *dVMAT* mutant in LD or DD conditions (Figure

3-8). These results are consistent with the idea that DVMAT expression in a single aminergic

regulatory system may not be sufficient to rescue the rhythmicity defect of the *dVMAT* mutant.

Normal circadian rhythmicity requires the expression of DVMAT in multiple aminergic cell types

Given the failure of any single aminergic Gal4 driver to rescue rhythmicity of the *dVMAT*

mutant, it seemed likely that multiple aminergic systems might be required for normal circadian

behavior. We therefore examined activity rhythms in *dVMAT* mutants expressing DVMAT under

the control of combinations of Gal4 drivers (Tdc + TH, Tdc + TrH, or TH + TrH) to restore

aminergic function in multiple different cell types. Interestingly, the arrhythmic phenotype of

the *dVMAT* mutant in DD was rescued only with simultaneous expression of DVMAT in at least

two classes of aminergic neurons (Figure 3-8 A-C). Expression of DVMAT in a mutant

background under control of any of the three possible combinations of drivers (Tdc + TH, Tdc +

TrH or TH+TrH) resulted in percent rhythmicity values (Figure 3-8 B) similar to those of wild-

type controls and average RIs (Figure 3-8 C) that were significantly higher than those of *dVMAT*

nulls or mutants carrying DVMAT and a single Gal4 driver (see Discussion).

DISCUSSION

In previous studies, genetic or pharmacological manipulation of individual aminergic systems has been the method of choice for determining their functions. Here I employed a different approach, exploiting the broad requirement of the vesicular monoamine transporter (VMAT) for vesicular release of neurotransmitter synaptic transmission in all aminergic neurons. My colleagues and I used the *dVMAT* null mutant to globally ablate exocytotic amine release, and then restored vesicular storage of individual amines or multiple systems using transgenic rescue techniques. Our results provide a detailed dissection of the functions of individual or combinations of aminergic neurotransmitters in the regulation of Drosophila behavior. Although certain Drosophila behaviors rely predominantly on a single aminergic system, others require multiple aminergic systems that may function redundantly or in concert in the coordination of behavior. Similar regulatory interactions may occur in mammals, for which the interplay between aminergic systems remains poorly understood (GAINETDINOV and CARON 2003; LEGGIO *et al.* 2009). In the sections below, we discuss in greater detail Drosophila behaviors which: 1) depend primarily on a single regulatory system, 2) require two or more aminergic systems that function redundantly or in a complementary manner or 3) behaviors that appear to require two or more systems working in concert. We emphasize that loss of DVMAT and the genetic experiments we have performed pertain only to standard exocytotic release of amines via secretory vesicles. It remains possible that other, novel types of release are active in the *dVMAT* mutant and important for some developmental processes as we discuss below.

Octopamine may be sufficient to rescue some behaviors

We find that expression of DVMAT via *Tdc2-Gal4* alone is sufficient to rescue the larval

lethality of the *dVMAT* mutant that occurs under standard culture conditions, indicative of a

developmental role for OA and possibly TA. This result also suggests that the vesicular release

of DA and 5-HT may be dispensable for larval development and adult survival, an observation

that is surprising given the fundamental role of DA in many physiological processes, and the

lethal effects of genetic (*pale*) (KOBAYASHI *et al.* 1995) or pharmacologic inhibition of tyrosine

hydroxylase activity (NECKAMEYER 1996; PENDLETON *et al.* 1996). Similarly, it has been

proposed that 5-HT has a critical role in fly development (COLAS *et al.* 1999; SCHAERLINGER *et

al.* 2007; SEDORE WILLARD *et al.* 2006; SYKES and CONDRON 2005). In contrast to these results,

it has been shown that mutants of aromatic L-amino acid decarboxylase/Dopa decarboxylase

(AADC/DDC) – which converts 5-hydroxy-L-tryptophan (5-HTP) to 5-HT (HODGETTS and

O'KEEFE 2006) – and flies with increased or decreased 5-HT survive beyond third instar larval

stages (BUDNIK *et al.* 1986; DAUBERT *et al.* 2010; NECKAMEYER 2010; SYKES and CONDRON

2005). Moreover, a recent study showed that *pale* mutants lacking DA synthesis in the nervous

system (but not cuticle-forming tissue) survived to adulthood, despite observed deficits in

phototaxis, arousal, and avoidance of shock-associated odor (RIEMENSPERGER *et al.* 2011).

Similarly, we find that flies unable to store or exocytotically release DA and 5-HT can survive

through larval and pupal development and eclose as viable adults. We suggest that the severe

developmental phenotype of the 5HT2Dro mutant may reflect a requirement for non-vesicular

release of 5-HT (SCHAERLINGER *et al.* 2007). In addition, consistent with (RIEMENSPERGER *et al.*

2011), we find that the neuronal storage and exocytotic release of DA is not required for

development. By contrast, other aspects of the phenotype of the *dVMAT* mutant and that reported

by Riemensperger *et al.* (2011) may differ. Future comparative studies of these mutants may

allow a dissection of the respective roles for standard vesicular release of DA versus other non-exocytotic mechanisms.

Similar to larval survival, the larval locomotion deficits of the *dVMAT* mutant can be rescued using either *da-Gal4* or, more specifically, *Tdc2-Gal4* to express DVMAT in OA+TA neurons. Conversely, although DA and 5-HT application may modulate firing patterns at the neuromuscular junction (COOPER and NECKAMEYER 1999; DASARI and COOPER 2004), we find that expression of DVMAT using *Ddc-*, *TH-* or *TrH-Gal4* does not significantly rescue the *dVMAT* larval locomotion phenotype. In addition, we find that feeding larvae the neurotransmitter OA, but not DA or other amines, to *dVMAT* mutants can partially rescue larval locomotion. At present we cannot rule out the possibility that OA is absorbed more readily than other amines. This caveat aside, our data are consistent with the critical role for OA in regulating motor behaviors in many invertebrates, in part by initiating the central pattern generators (CPGs) for crawling, walking, and flying (BAUDOUX *et al.* 1998; HASHEMZADEH-GARGARI and OTTO FRIESEN 1989; ORCHARD *et al.* 1993; RAMIREZ and PEARSON 1991; ROEDER 1999; SOMBATI and HOYLE 1984). Once initiated by OA or other modulatory neurotransmitters, CPGs can propagate a stereotypic behavior independent of sensory input (GRILLNER 2003; MARDER and BUCHER 2001).

In the Drosophila larva, both TA and OA have been proposed to have opposing effects on locomotion, with OA stimulating and TA possibly inhibiting the CPG (FOX *et al.* 2006; SARASWATI *et al.* 2004). We do not detect a decrease (or increase) in locomotion when either *dVMAT* mutant or wt larvae are fed TA. It is possible that pharmacologic manipulation of TA is less robust than the previously described genetic approach and larval are unable to absorb TA or

perhaps that a distinct, sensitized background will be needed to detect the behavioral effects of feeding TA.

The apparent dominance of OA versus either DA or 5-HT for the behaviors we have studied here may be surprising but is consistent with a range of other studies in invertebrates demonstrating the fundamental importance of OA in a variety of basic behaviors (BICKER 1999; CHASE and KOELLE 2007; ROEDER 1999; SCHEINER *et al.* 2006; VERLINDEN *et al.* 2010). It is nonetheless possible that we failed to detect the importance of 5-HT in some cases because of the limited rescue of 5-HT levels using the relatively weak *TrH-Gal4* driver. However, for both survival and larval locomotion, the contribution of serotonin was also tested using the *Ddc-Gal4 driver,* which drives expression in both DA and 5-HT neurons, and also failed to rescue the *dVMAT* phenotype. In addition, DVMAT expression using *TrH-Gal4* significantly altered both startle and circadian behaviors, thus demonstrating functional effects of *UAS-DVMAT* expression in 5-HT cells using this driver despite the limited biochemical rescue of 5-HT levels. We also note that the degree to which *TrH-Gal4* rescued 5HT levels was similar to the rescue of OA by *Tdc2-Gal4*.

We observe a small but significant loss of DA neurons in the *dVMAT* mutant larvae as well as adults (LAWAL *et al.* 2010). We cannot rule out the possibility that the loss of DA neurons mitigated the ability of DA feeding to rescue larval locomotion. It is also possible that additional changes in the aminergic neuropil could mitigate some aspects of our chemical rescue data. Changes in the branching pattern of serotonergic processes have been observed in the *Ddc* mutant (BUDNIK *et al.* 1989) and in larval brains exposed to excess exogenous 5-HT, (SYKES AND CONDRON 2010) and serotonin may generally function as a trophic agent (DAUBERT

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and CONDRON 2010). In addition, under some conditions, exogenous octoamine can increase the number of filopodia or synaptopods seen on octopaminergic processes (KOON *et al.* 2011

). Similar developmental changes caused by abnormal release in the dVMAT mutants could block its ability to respond to exogenous neurotransmitter and perhaps genetic rescue. In contrast, since the genetic rescue paradrigm we used here was constitutive and throughout development, this issue is less likely to be confound experiments using the UAS-*DVMAT* transgene, which make up the bulk of our data.

Complementary aminergic circuits

     dVMAT mutant adults show deficits in motor behavior, but the adult *dVMAT* phenotype is more complex than that of the larva. Adult flies have periodic bouts of flying, walking and relatively stationary grooming and all of these behaviors may undergo aminergic regulation. Startle-induced locomotion is likely to be particularly important for the escape of adult flies from predators and survival and DA has been previously shown to play an important role for this behavior in Drosophila (LEBESTKY *et al.* 2009). In addition, photostimulation of dopaminergic neurons triggers locomotion (LIMA and MIESENBÖCK 2005) and inhibition of tyrosine hydroxylase decreases locomotion (PENDLETON *et al.* 2002). DA and 5-HT neurons appear to regulate rhythmic flight activity (BANERJEE *et al.* 2004) and application of aminergic drugs to headless flies stimulates motor behaviors (YELLMAN *et al.* 1997). Our data are consistent with a model where aminergic circuits complement one another to regulate startle-induced locomotion. We note that *dVMAT* mutants show mildly elevated locomotion in the open field (SIMON *et al.* 2009) and at baseline in the startle assay we have used here. For both of these assays, it is

possible that handling of the flies increased motor behavior, since true baseline activity recorded

over the course of many hours revealed a small *decrease* in the *dVMAT* mutant relative to wt.

The *dVMAT* mutant showed a robust decrease in startle-induced locomotion using a puff

of air to stimulate locomotion as previously described (LEBESTKY *et al.* 2009). Expression of

UAS-DVMAT using *Tdc2-Gal4* rescued some of these deficits including peak velocity and

distance traveled in response to startle, clearly demonstrating an important role for OA and

perhaps TA in some aspects of adult startle response and locomotion. These data are consistent

with the proposed role for OA in both arousal and the fight-or-flight response in other insects

(ADAMO *et al.* 1995; BACON *et al.* 1995; RIND *et al.* 2008; STERN 1999).

Although the results were less dramatic than those seen using *Tdc2-Gal4*, expression of

DVMAT using *TrH-Gal4* revealed significant rescue effects in the absence of *Tdc2-Gal4*.

Effects associated with *Trh-Gal4* were detectable during return to "baseline" activity after startle.

The behavior of mutants expressing DVMAT with *TrH-Gal4* suggest that 5-HT neurons may be

important for regulating at least some parameters of the post-startle behavior, including tau and

asymptotic velocity. We suggest that the complexity of adult locomotor behavior may require

several complementary aminergic systems to regulate specific aspects of this behavior; for

example, OA may initiate a fight-or-flight response but other amines are responsible for shaping

the appropriate response after initiation. In contrast, the relatively simple larval locomotor

behaviors tested here appear to require regulation primarily if not exclusively by OA.

Similar to larval locomotion, expression of *UAS-DVMAT* in OA cells (using *Tdc2-Gal4*)

and feeding OA rescued the infertility of adult *dVMAT* females. Previous genetic studies have

demonstrated a critical role for OA in female fertility (GRUNTENKO *et al.* 2007; HARDIE *et al.*

2007; LEE *et al.* 2003; MONASTIRIOTI 2003; MONASTIRIOTI *et al.* 1996; NECKAMEYER 1996;

SEDORE WILLARD *et al.* 2006). OA is likely to act at several sites in the female reproductive system to enable egg-laying, and activation of different types of OA receptors may mediate contraction of the musculo-epithelial net surrounding the ovary, relaxation of the oviduct, and lubrication of the oviduct via epithelial cells that line the lumen (LEE *et al.* 2009; LEE *et al.* 2003; MIDDLETON *et al.* 2006; RODRÍGUEZ-VALENTÍN *et al.* 2006).

A possible role for 5-HT and/or DA in female fertility and egg development has been suggested previously (NECKAMEYER 1996; SEDORE WILLARD *et al.* 2006). We find that 5-HT is neither required nor sufficient to rescue the fertility defect of the *dVMAT* mutant (See also SIMON *et al.* 2009). However, consistent with a possible role for DA in early development, fertility and ovary size in the *dVMAT* mutant are modestly increased when DVMAT expression is restored solely in dopaminergic neurons. Ovary size may increase because dopamine enhances or accelerates oocyte development (SEDORE WILLARD *et al.* 2006). In the absence of octopaminergic signaling to allow their release, a larger number of late-stage oocytes may be retained in the ovary.

In contrast to the characterization of female fertility, the contribution of biogenic amines to normal male sexual function has remained relatively obscure (GREENSPAN 2000; HALL 1994; LASBLEIZ *et al.* 2006; YAMAMOTO *et al.* 1997). One recent report suggests that the 5-HT7 receptor is involved in male courtship (BECNEL *et al.* 2011). Overexpression or elimination of one aminergic system at a time has also demonstrated possible dopaminergic effects on courtship conditioning, a learned behavior (NECKAMEYER 1998a), and aberrant male-male courtship (LIU *et al.* 2008), but only one additional report has suggested that standard male-female courtship also might be affected (ALEKSEYENKO *et al.* 2010). Similarly, the effects of OA on courtship have been limited to the interface between courtship and aggressive behaviors rather than

courtship per se (CERTEL *et al.* 2007). Using genetic rescue of individual aminergic systems in the *dVMAT* mutant, we demonstrate a role for both DA and OA in normal male sexual activity. Interestingly, the expression of *UAS-DVMAT* using either *TH-Gal4* or *Tdc2-Gal4* rescues the *dVMAT* mutant male courtship phenotype to a similar degree. We speculate that DA and OA may serve redundant or overlapping roles in activating male courtship. Mechanistically, it is possible that DA and OA inputs converge upon a similar common pathway or stimulate courtship via distinct pathways. We speculate that roles for both DA and OA in male courtship may not have been previously obvious since signaling via DA or OA, but not both, were disrupted in other studies (CERTEL *et al.* 2007; NECKAMEYER 1998b).

Possible cooperative effects between aminergic systems

Our results suggest that interactions between aminergic systems may be particularly important for the regulation of circadian rhythms. An earlier study indicated that 5-HT is required for light input to the clock circuitry via the 5-HT1B receptor and glycogen synthase kinase 3β signaling (Yuan et al., 2005). More recently, it has been shown that flies with reduced neural DA exhibit defects in circadian light sensitivity and/or show weak free-running rhythmicity (i.e., in DD; Hirsh et al., 2010). We find that *dVMAT* mutants lacking vesicular release of 5-HT, DA and OA show decreased activity in the DAMS monitor (data not shown) and exhibit an altered profile of daily activity in LD. Specifically, there is an unusually high percentage of night-time activity and the morning bout of activity is proportionally decreased relative to the wild-type profile, or missing entirely in many mutants (not shown). A selective effect on morning versus evening activity is of interest because different groups of clock neurons are postulated to control the two bouts of activity (NITABACH and TAGHERT 2008), although

there is still ongoing debate about the precise composition of the evening and morning circadian

oscillators (e.g., SHAFER and TAGHERT 2009; SHEEBA *et al.* 2010). In addition to the LD

phenotype, most *dVMAT* individuals – even those that exhibit bimodal activity in LD – are

weakly rhythmic or arrhythmic in free-running conditions. Thus, modulation by biogenic amines

is also essential for free-running rhythmicity.

As both DA and 5-HT are required for normal circadian light sensitivity, a deficit in this

process might contribute to the phenotype of *dVMAT*. However, neuronal PER cycling can be

synchronized to LD and persists in DD in the *dVMAT* mutant (data not shown), suggesting that

aminergic circuits modulate clock output rather than directly affecting the circadian pacemaker.

It remains possible that amines contribute to the so-called "positive masking" response to lights-

on – a clock-independent direct stimulation of locomotor activity (RIEGER *et al.* 2003; WHEELER

et al. 1993) – as most mutant individuals do not exhibit the short, light-induced bout of activity

that is observed at the beginning of day in wild-type flies. Of note, increased activity is observed

in mutant individuals in response to the lights-off signal, so the effect on masking may be

selective for the lights-on signal. To our knowledge, it is not known whether distinct circuits

control responses to lights-on and lights-off in Drosophila.

Surprisingly, we were unable to detect partial rescue effects for rhythmicity with any

single driver. Rather, restoration of DVMAT expression in two classes of aminergic neurons was

required to detectably rescue circadian behavior. One trivial explanation for this observation

would be that the methods we used to quantify circadian behavior were less sensitive than those

used for other behaviors. We think this is unlikely, and in some cases, single drivers did indeed

show a detectable effect, but worsened rather than rescued the mutant phenotype. We speculate

that the apparent requirement for at least two systems may reflect an unexpected cooperativity

between aminergic circuits that regulate circadian rhythms. Although the sites of these interactions are not clear, it is conceivable that aminergic inputs to the clock neurons are involved. It is known, for example, that there are functional 5-HT (HAMASAKA and NÄSSEL 2006) and OA (KULA-EVERSOLE *et al.* 2010) inputs to the small and large ventral lateral clock neuron (LNv) populations. Further efforts to map the circuitry of the adult fly brain may reveal additional potential sites of interaction.

Another interesting aspect of these data is that any combination of two Gal4 drivers (TrH + TH, Tdc + TH or Tdc + TrH) was sufficient to rescue the circadian phenotypes of the *dVMAT* mutant (with the exception of the RI value for LD entrainment of the Tdc + TrH combination which failed to reach statistical significance). This demonstrates that two of the three aminergic systems suffice for rhythmicity, and that no one specific combination is absolutely required for circadian behavior. This result contrasts with that of some other behaviors we examined in which particular systems were necessary-and-sufficient (larval locomotion, survival, female fertility), but is similar to male sexual behavior in which both OA and DA were capable of stimulating courtship and rescuing fertility. Our data suggest the possibility that there are redundant aminergic inputs to the circuitry regulating clock-based as well as courtship circuitries. In the case of the clock circuits, previous results indicate plasticity in the clock neurons regulation of locomotor activity (SHEEBA *et al.* 2010), and network properties of the circadian circuitry may permit flexibility such that the absence of one set of synaptic interactions does not disrupt behavior.

The possibility that two aminergic systems can control the same behavior has important implications in the fly as well as mammalian systems. In mammals, the prediction that DA circuits were responsible for psychostimulant-based reward behavior has matured into a view

that DA as well as NE and 5-HT are involved (TORRES *et al.* 2003). Similarly, some behaviors

in the fly may undergo control by multiple regulatory circuits, whose input would vary

depending on shifting environmental conditions or internal states.

FIGURES

Figure 3-1. Protein expression and neurotransmitter content in genetically rescued *dVMAT* mutants.

(A) Western blots show expression of the HA-tagged DVMAT transgene in *dVMAT* mutant (-/-) expressing *UAS-DVMAT* with the indicated drivers, *TH-Gal4* (TH), Tdc2-Gal4 (Tdc), *TrH-Gal4* (TrH), *Ddc-Gal4* (DDC), and *da-Gal4* (ubiq^{-5HT}). Note that the *UAS-DVMAT* transgene (UAS) shows "leaky" expression in the absence of driver.
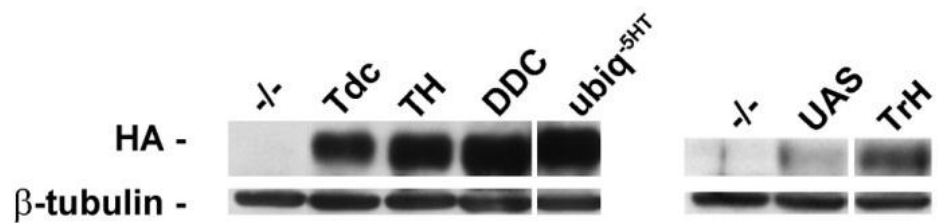
Figure 3-2. Neurotransmitter content in genetically rescued *dVMAT* mutants.

Content of DA (A, B), 5-HT (C, D) and OA (E, F) in the *dVMAT* mutant (-/-) expressing *UAS-DVMAT* with the indicated drivers (A, C, E); wt controls are shown for comparison (B, D, F). Note that the y axes differ across panels. HPLC with electrochemical detection was used for homogenates of either adult heads (5-HT and DA) or adult brains (OA). Non-parameteric ANOVA was used for analysis (Dunn's post hoc, $*p<0.05$ as indicated) since some mutant values were undetectable.
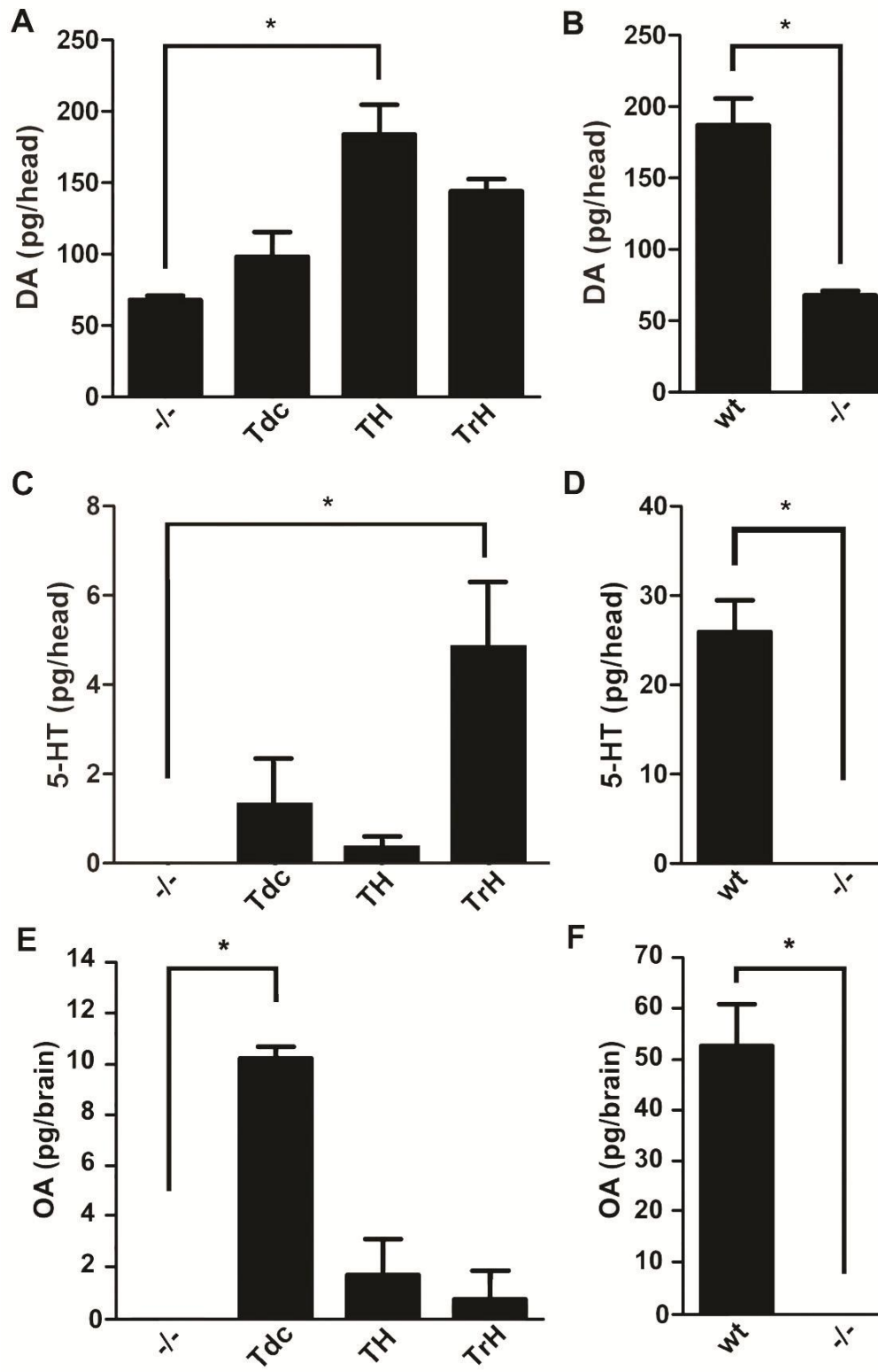
Figure 3-3. Aminergic cell counts in *dVMAT* mutants and rescue of density dependent lethality.

(A, B) *dVMAT* mutants possess a reduced number of dopaminergic neurons of the DL2 cluster but are otherwise comparable to wt. (A) The arrangement of DA neuron clusters are pictured in wild-type larva with the broken rectangle indicating the DL2 cluster. Inset shows a representative image of DL2 cluster in the wild-type (wt) and *dVMAT* mutant (-/-). (B) Quantitation of each cluster (white bars: wt; black bars mutant). Differences between the number of octopaminergic (C, D) or serotonergic (E, F) neurons in wt (n=9) versus mutant (n=12) are not detectable for each indicated cluster. Scale bars A, B, C: 50 um; A inset: 10 um. (G) Survival Assays. Under standard culture situations (500-1000 progeny/bottle), approximately 6% of the homozygous *dVMAT* null mutant (-/-) progeny survive. Expression of a DVMAT transgene in DA or 5-HT cells using *TH-Gal4* (TH), *TrH-Gal4* (TrH), or *Ddc-Gal4* (Ddc) respectively, do not rescue the survival deficit, whereas expression of DVMAT using *da-Gal4* (ubiq^{-5HT}) or *Tdc2-Gal4* (Tdc) significantly rescues lethality under standard culture conditions (1-way ANOVA followed by Bonferroni's Multiple Comparison Test). The raw data used for panel G is shown in Figure 3-4.
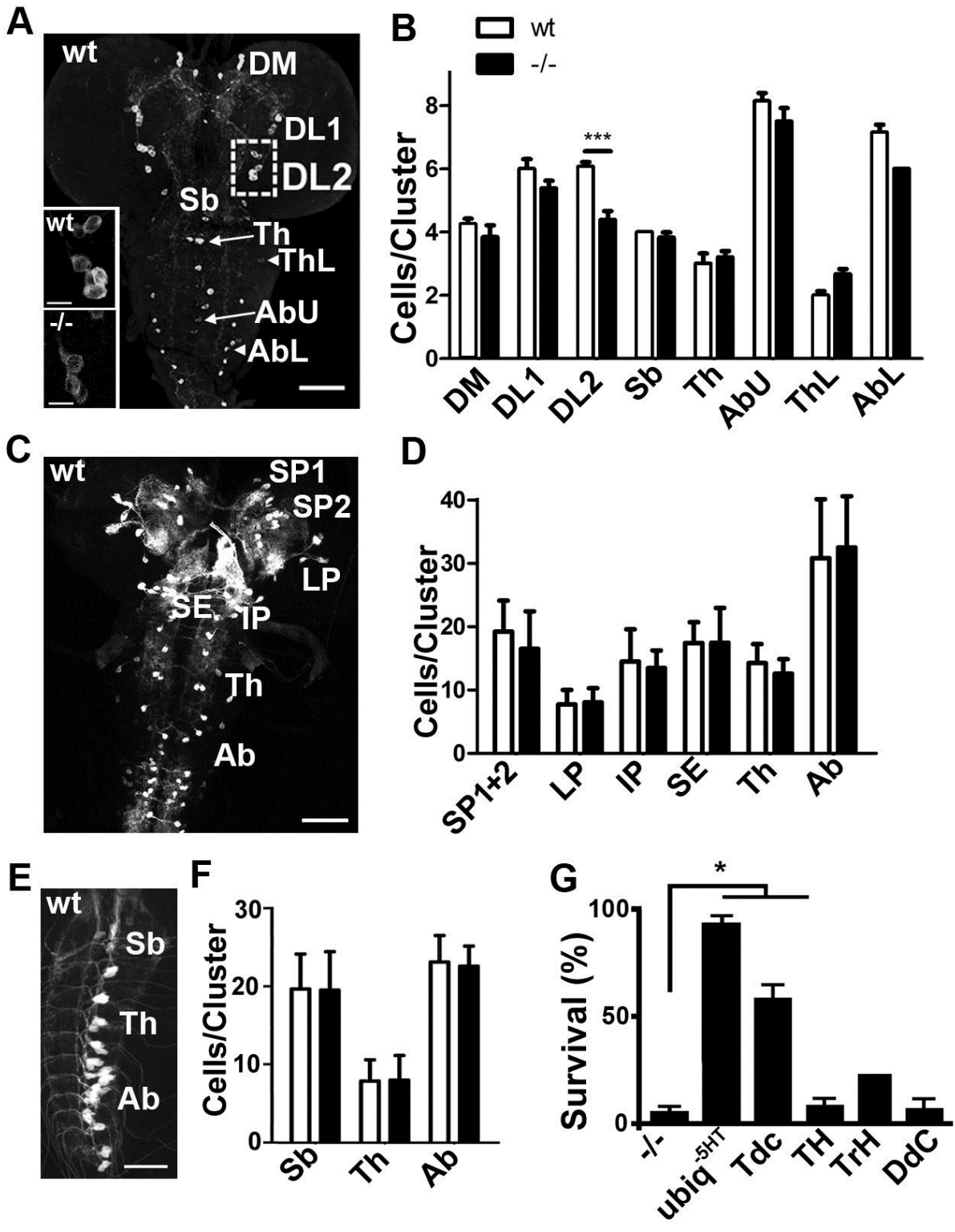
Figure 3-4. Homozygous survival plotted as a function of population density, and with *dVMAT* mutants expressing *UAS-DVMAT* using the indicated drivers.

(A) Survival of homozygous *dVMAT* progeny (-/-) are plotted as a function of population density in black. Data for *dVMAT* mutants expressing *UAS-DVMAT* using the *Ddc-Gal4* are shown in blue. Rescue of *dVMAT* using *TH-Gal4* (B), *TrH-Gal4* (C), *Tdc-Gal4* (D), and *daughterless-Gal4* (E) is indicated. Second-order polynomial trendlines are displayed as solid lines.
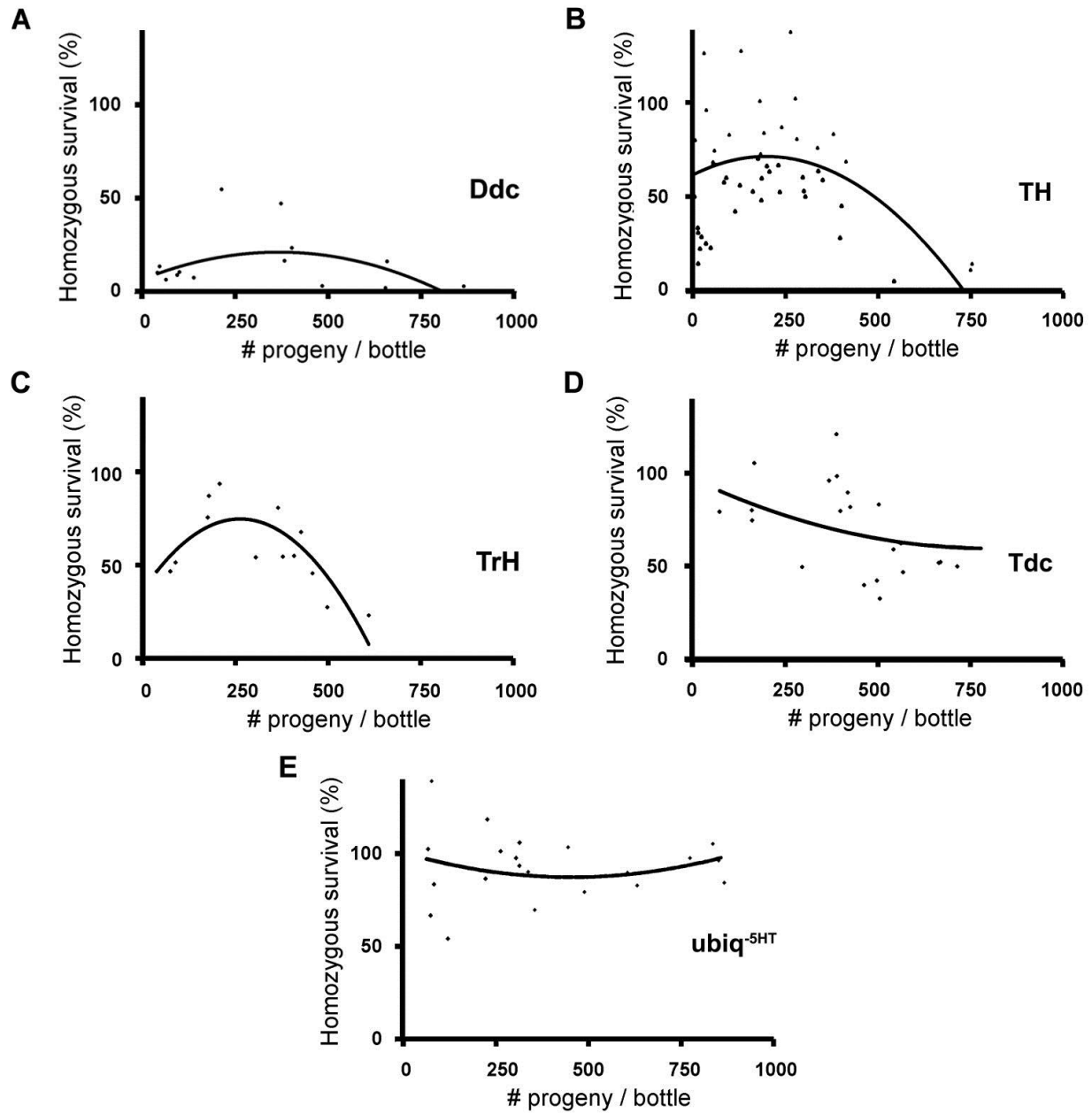
Figure 3-5. Octopamine rescues larval locomotion.

(A) Expression of DVMAT using *Tdc2-Gal4* but neither *TH-Gal4* (TH) nor *TrH-Gal4* (TrH) rescue larval locomotion to levels significantly higher than the *dVMAT* mutant (-/-), albeit less than wt (1-way ANOVA, *$p<0.05$, Bonferroni Post Test to compare indicated columns). Feeding octopamine (B) but not tyramine (C) or other amines (not shown) increased larval locomotion in the *dVMAT* mutants.
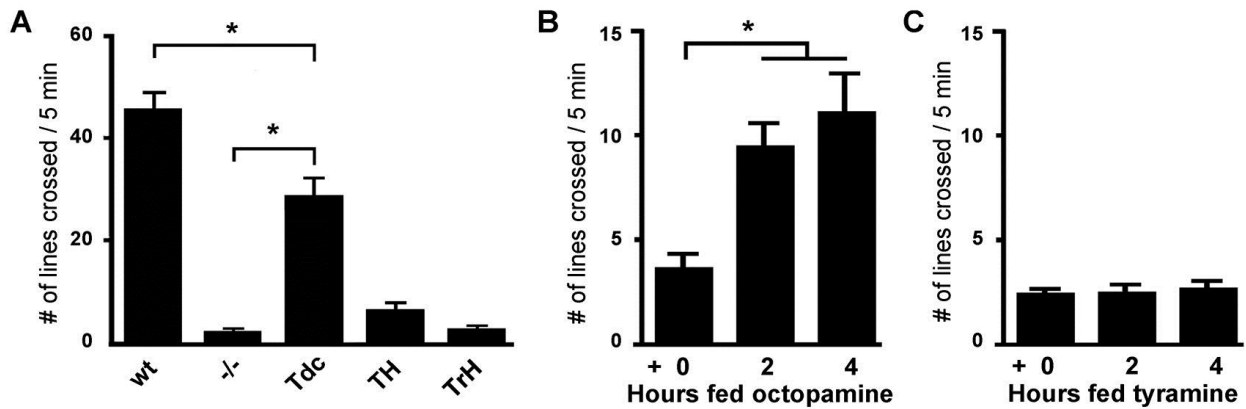
Figure 3-6. Dopamine and octopamine contribute to fertility and sexual behavior.

(A) Expression of DVMAT using *da-Gal4* (ubiq^{-5HT}) or *Tdc2-Gal4* (Tdc) rescues the *dVMAT* female fertility defect (Fisher's Exact Test, *$p<0.05$). Rescue using *TH-Gal4* (TH) was less robust but significant (*$p<0.05$); in contrast to Tdc rescue, fertility is not rescued up to wt levels. Flies expressing DVMAT in serotonergic (TrH) neurons remained infertile. (B-C) *dVMAT* mutants retain more oocytes than wt controls (see also Simon el al, 2008). Expression of DVMAT using *Tdc-Gal4* rescues this deficit (1-way ANOVA, *$p<0.05$, Bonferroni Post Test). Expression using *TH-Gal4* (TH) shows a trend toward retention of more oocytes than the mutant. (D) Expression of DVMAT using *da-Gal4* (ubiq^{-5HT}), *TH-Gal4* (TH), or *Tdc2-Gal4* (Tdc) rescues the *dVMAT* male fertility defect (Fisher's Exact Test, *$p<0.05$). (E) Male courtship is reduced in the *dVMAT* mutant (-/-) and partially rescued using either *Tdc2-Gal4* or *TH-Gal4* (1-way ANOVA, *$p<0.05$, Bonferroni Post Test) but not *TrH-Gal4*.
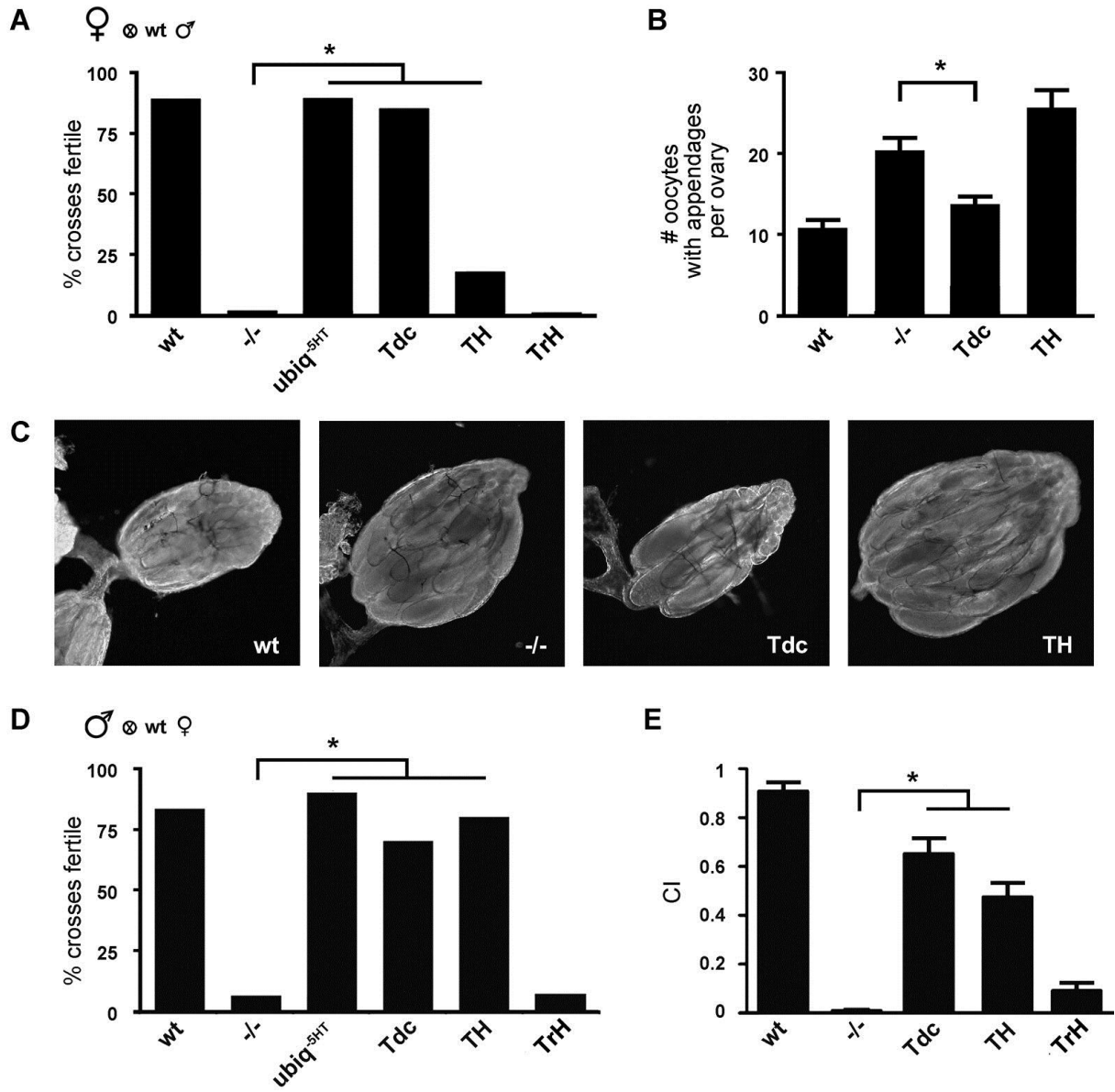
Figure 3-7. Restoration of DVMAT in individual aminergic systems rescues selected aspects of adult startle-induced locomotion.

(A) Negative geotaxis assay. Given 15 sec to climb after a mechanical startle, flies with DVMAT restored in OA+TA neurons via *Tdc2-Gal4* have improved startle responsiveness compared to *dVMAT* mutants. Rescue with *TH-Gal4* or *TrH-Gal4* did not display improved performance (1-way ANOVA, *p<0.05, Bonferroni Post Test; n = 23 tubes for wt, n=15 for -/-, n=8 for ubiq^{-5HT}, n=13 for Tdc, n=14 for TH, n=10 for TrH). (B-H) Phenotypic characterization of startle response to successive air puffs. n = 24 tubes per genotype. (B) Peak velocity – Baseline velocity after air puffs is rescued in flies with DVMAT restored using *Tdc2-Gal4*. Traces show selective restoration of DVMAT using *Tdc2-Gal4* (C), *TH-Gal4* (D) or *TrH-Gal4* (E). (F) DVMAT expression in OA+TA neurons using *Tdc2-Gal4* rescues distance traveled after startle, computed by integrating the area under the post-puff curve, after subtracting the pre-puff baseline. (G) Post-startle rate to acclimation is reported as the post-startle decay constant tau, and is rescued using either *TH-Gal4* or *TrH-Gal4*. Rescue using *Tdc2-Gal4* differs from the mutant but is also higher than wt, suggesting a prolongation of post-startle acclimation. (H) Asymptotic velocity represents the estimated final settle velocity after the puff startle. *dVMAT* flies remain hyperactive after startle. The elevated asymptotic velocity is rescued with DVMAT expression in 5-HT neurons (Kruskal Wallis ANOVA followed by Mann Whitney U test: *p<0.05).
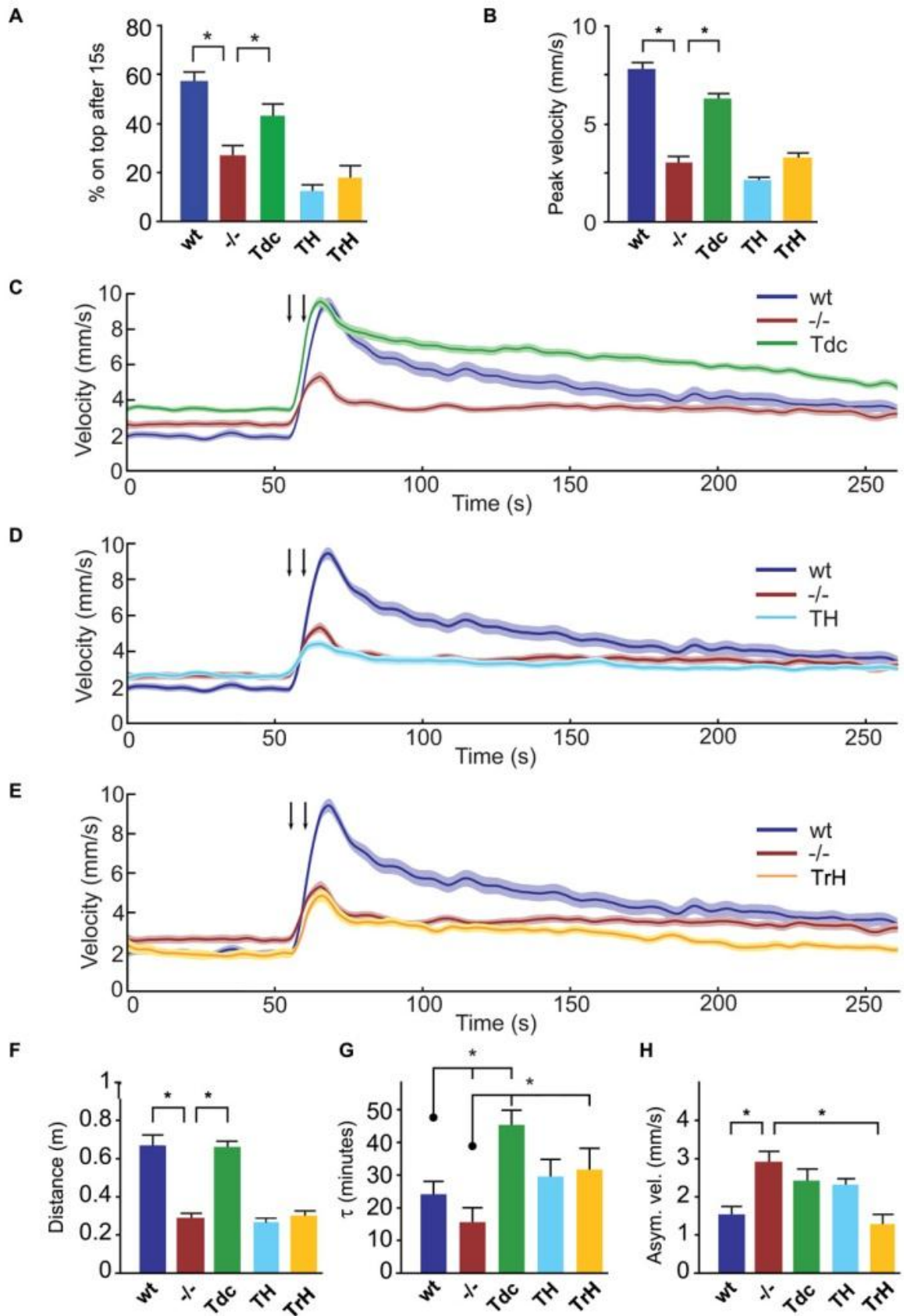
Figure 3-8. Arhythmicity of the *dVMAT* mutant in constant darkness (DD) can be rescued using pairs of aminergic drivers.

Rhythmicity of the mutant is rescued by simultaneous expression of wild-type DVMAT in two of the three different aminergic neuronal populations, but not using a single driver. (A) Representative DD actograms for each genotype. (B-C) Histograms indicating the percentage of flies that showed rhythmic behavior (rhythmicity, B) and the corresponding average rhythmicity index (RI value). (C) Flies with DVMAT expression in multiple aminergic neuronal subsets were significantly different from the mutant suggesting phenotypic rescue (Kruskal-Wallis non-parametric ANOVA, Dunn's Multiple comparisons test, $*p<0.05$).
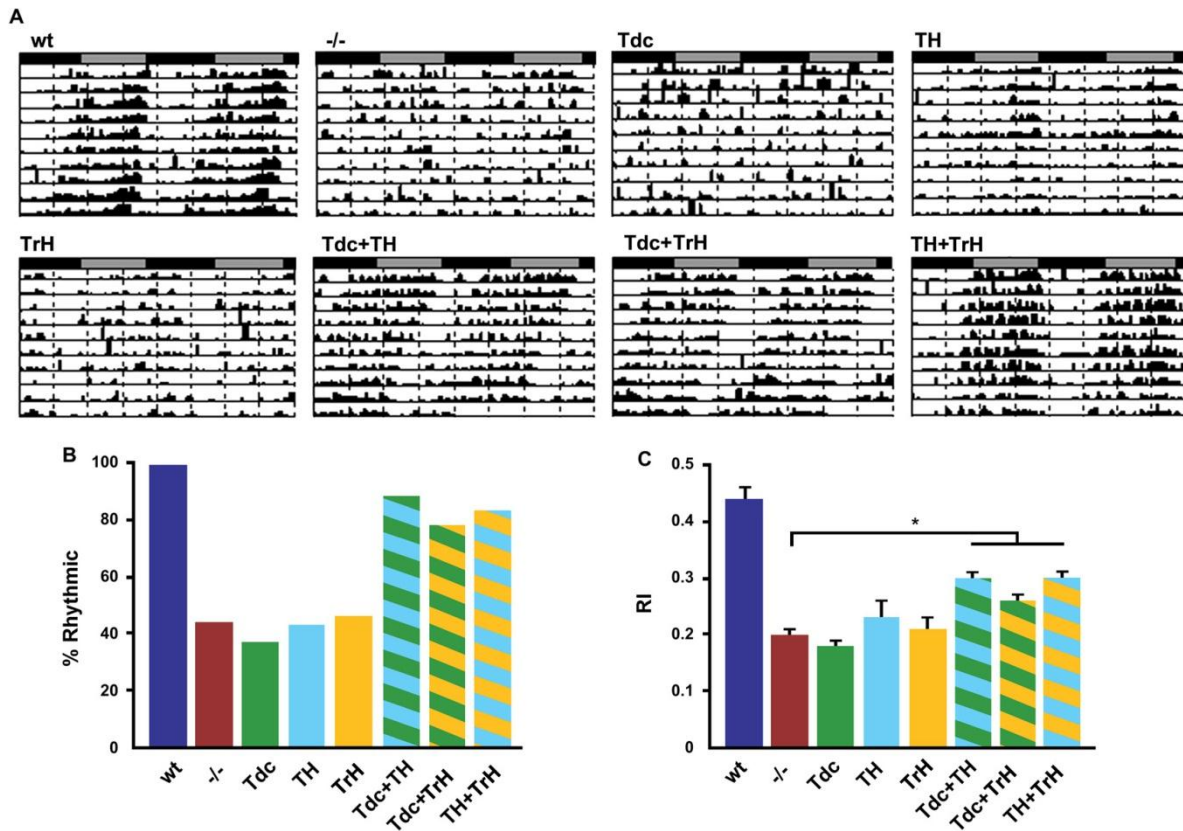
CHAPTER FOUR:

Temporally restricted requirements
of vesicular neurotransmitter transporters
differ in aminergic systems and GABAergic systems

SUMMARY

Many proteins have temporal requirements for regulating behavior. The *Drosophila* model organism offers multiple options for inducible transgene expression. Here, the Gal80ts system was used to probe the temporal requirement for two vesicular neurotransmitter transporters: vesicular monoamine transporter (VMAT) and vesicular GABA transporter (VGAT). By confining VMAT expression to either early development or late adulthood, I find that monoaminergic signaling during either time period was sufficient to rescue one behavioral output: fertility. In contrast VGAT expression during development is required for rescue of lethality. When the Drosophila ortholog of VGAT (referred to here as dVGAT) is expressed selectively during development, lethality is rescued, enabling study of the role of GABA release and inhibitory neurotransmission in regulating visual behavior. My collaborators and I show that reduced GABA release does not compromise the active optomotor control of wide-field pattern motion. Conversely, reduced dVGAT expression disrupts normal object tracking and figure–ground discrimination. These results demonstrate that visual behaviors are segregated by the level of GABA signaling in flies, and exemplify the utility of temporally confining vesicular neurotransmitter transporter expression using the genetic toolkit available in *Drosophila.*

INTRODUCTION

The previous chapter examined the cooperative or redundant roles of monoaminergic neurotransmitter input onto circuits using targeted transgene expression in the spatial dimension. This chapter examines temporal requirement for monoaminergic input by targeted gene expression in the dimension of time.

The GAL80^{ts} inducible genetic system was used in conjunction with the pre-existing GAL4/UAS system to induce vesicular neurotransmitter transporter expression in a stage-specific manner. The Gal80ts system is one tool in the *Drosophila* genetic toolbox which allows temporally control of transcription and subsequently protein expression. This temperature-sensitive system prevents transgenes of interest to be transcribed at "permissive" temperatures (18°C) when the transcriptional repressor GAL80 is active. The tetracycline-dependent transactivator (Tet-off) system (BELLO *et al.* 1998) offers another alternative to temporal control of gene expression. Used in combination with existing GAL4/UAS lines, it also adds temporal control on top of spatial specificity provided by tissue-specific GAL4 drivers (BRAND and PERRIMON 1993). When larvae are raised in media containing a tetracycline, e.g. doxycycline (DOX), the tetracycline prevents binding of the tetracycline-controlled transactivator protein, thus silencing the responder construct. Expression of the responder construct is induced when larvae are moved to a DOX-free environment. The Tet-off system has the advantage of providing a higher level of expression than the Geneswitch system (FORD *et al.* 2007) and does not rely on temperature to switch on transgene expression as in the GAL80^{ts} system. New versions of the Tet-off system have corrected for leaky expression in the target constructs (FORD *et al.* 2007), however the system must also take into account the kinetics of DOX clearance or

variation in transcriptional activation (BELLO *et al.* 1998). The Gal80ts system was chosen because transgene expression can be easily induced by shifting organisms from one temperature environment to another, and experiments do not require production of food preparations with various concentrations of an inducing drug.

Restricting monoaminergic circuit in time dimension

Previous studies in the mouse suggest that altered aminergic signaling in the embryo lead to lasting changes in the neural circuitry (MURPHY *et al.* 2001). Past studies have shown that reduced serotonin transporter (SERT) expression during development results in abnormal affective and anxiety-like behaviors in adult rodents (Ansorge et al., 2004). These studies suggest that perturbations which alter monoamergic signaling during developmental have long-lasting effects on complex behaviors. Although plasma membrane transporters, such as SERT, have a developmental requirement, it is unclear whether other transporters which regulate neurotransmission at monoaminergic synapses are equally sensitive. At the control point for regulating neurotransmitter content in a vesicle, VMATs regulate transmission differently than reuptake transporters and may have a different mechanism for adapting to changes in expression level.

In this chapter, I employ the Gal80ts inducible system to two neurotransmitter systems. In part 1, I use the Gal80ts system to repress expression of *Drosophila* vesicular monoamine transporter (dVMAT) during early development and examine the long-term effects on an adult behavior. In Part 2, I use the Gal80ts system to repress expression of the vesicular GABA transporter selectively during adult stages and find roles for GABA in figure ground detection.

Restricting GABAergic circuit in temporal dimension

Two types of neurotransmitter transporters are required for the storage and recycling of gamma amino butyric acid (GABA), and both are likely to contribute to the regulation of GABAergic neurotransmission. The plasma membrane GABA transporters (GATs1-3) remove neurotransmitter from the synaptic cleft after it is released (KANNER 2006; SCHOUSBOE 2000). A structurally distinct vesicular GABA transporter (VGAT) is required for its storage in synaptic vesicles (GASNIER 2004). In mammals, VGAT is required for the storage of both GABA and glycine (MCINTIRE *et al.* 1997; WOJCIK *et al.* 2006) and is also known as the vesicular inhibitory amino acid transporter (VIAAT) (SAGNÉ *et al.* 1997). Surprisingly, it is not known how changes in VGAT/VIAAT expression or activity may affect complex behaviors potentially dependent on GABAergic neurotransmission. With the possible exception of gamma hydroxy butyrate (GHB) (MULLER *et al.* 2002), no known drug specifically binds to or inhibits the function of VGAT/VIAAT, essentially prohibiting pharmacologic studies. The absence of available pharmacologic probes underscores the need for genetic models for in vivo analyses, but past studies of complex behavior have been relatively limited. In *C. elegans*, 25 of the 26 GABAergic neurons innervate muscle (SCHUSKE *et al.* 2004) and the phenotype of the VGAT mutant *unc-47* results from deficits at the neuromuscular junction rather than the central nervous system (BRENNER 1974; MCINTIRE *et al.* 1997; SCHUSKE *et al.* 2004). In mice, knockout of VGAT/VIAAT is lethal and homozygous mutants die between embryonic day 18.5 and birth (WOJCIK *et al.* 2006). GABAergic synapses in VGAT/VIAAT heterozygotes have electrophysiological properties similar to those of wild-type mice (WOJCIK *et al.* 2006) and it remains unclear whether the heterozygotes have a detectable behavioral phenotype.

To elucidate the role of GABA release in the function of the central nervous system and complex visual behavior, Julie Simpson and colleagues cloned and characterized the fly ortholog of the vesicular GABA transporter, herein referred to as *dVGAT*. The open reading frame (ORF) encodes a 1.7 kb protein that is 44% identical with human VIAAT/VGAT and similar to the previously identified *C. elegans* and mouse orthologs of VGAT (MCINTIRE *et al.* 1997; SAGNÉ *et al.* 1997). I showed that mutation of the *dVGAT* gene is lethal in the embryo. To examine the temporal requirements for dVGAT during development, I used an inducible expression system to rescue the developmental lethality of *dVGAT* and, in collaboration with Dawnis Chow, found that a decrease in adult expression of *dVGAT* compromises visual object detection.

Using the temperature-inducible GAL80ts system in the dVGAT animal, I show that temporal control of transgene expression can be used to assess requirements for GABA release in mutants that are normally unviable. These studies exemplify how tools available in *Drosophila* genetics can be used to bypass embryonic lethality to explore roles of inhibitory neurotransmission and act as a model for how inducible transgene expression system can be used to further tease apart temporal requirements of monoaminergic neurotransmission.

RESULTS

Temporal control of dVMAT-A transgene expression

In order to determine whether developmental deficits cause the adult mutant phenotype, the GAL80^{ts} inducible genetic system was utilized to induced *VMAT* expression in a stage-specific manner when used in conjunction with the pre-existing GAL4/UAS system. My characterizations of GAL80 control of *VMAT* expression have shown that flies must be shifted to "restrictive" (30$^{°}$C) temperatures for 20 hours to turn on protein expression, but once protein

75

expression is turned on, proteins are stable and do not turn off when switched to "permissive" temperatures (Data not shown).

Developmental expression of dVMAT not required for rescue of an adult behavior

In order to test whether developmental deficits cause the adult mutant phenotypes, I used the GAL80 inducible system to suppress VMAT expression during larval phases. VMAT expression during development is not required for rescue of viability (SIMON *et al.* 2009); thus it was possible to repress VMAT expression during early development without affecting survival. Flies were reared at 18°C during early development and shifted to 30°C 1-day post-eclosion (Figure 4-1 A). Western blot analysis of adult heads show that ubiquitously driven *dVMAT* expressed without the temperature-sensitive GAL80 have constitutive expression of VMAT regardless of changes in temperature (Figure 4-1 A, "const"). 1-day-old adults and 3-day-old adults reared continuously at 18°C show similar levels of expression as age-matched flies shifted to 30°C during the first 24hrs after eclosion and reared at 30°C for either 1 day or 3 days (Figure 4-1 A, "const"). In contrast, ubiquitously driven *dVMAT* co-expressed with the temperature-sensitive GAL80 repressor show temperature-dependent expression of VMAT (Figure 4-1 A, "Gal80^{ts}"). 1-day-old adults and 3-day-old adults reared continuously at 18°C do not express VMAT. At 18°C, GAL80 is active and represses transcription of *dVMAT*. When flies are reared at 18°C until eclosion, shifted to 30°C during the first 24 hours post-eclosion, and sacrificed 1 day later, flies show a moderate level of VMAT. Flies which are shifted at the same point of development but allowed to mature at 30°C until they are 3-days-old show stronger VMAT expression (Compare "18° to 30°C, 1d" to "18° to 30°C, 3d" in Figure 4-1 A).

Transgenic flies under inducible VMAT expression were then tested for female fertility, a

behavior previously described to be deficient in *dVMAT* mutants. At 18°C, 100% of wild-type

flies are fertile (Figure 4-1 B, "+/+") and 100% of *dVMAT* null mutants are infertile ("-/-").

Constitutively rescued flies (*w; dVMAT^{P1}/dVMAT^{P1}; UAS-VMAT-A, daughterless-GAL4/+*) are

fertile, though temperature appears to affect fertility rates. 32 percent of flies are fertile (Figure

4-1 B, "rescue 18°C"). This fertility rescue is not seen in flies expressing GAL80^{ts} (*w;*

dVMAT^{P1}/dVMAT^{P1}; UAS-VMAT-A, daughterless-GAL4/Tub-GAL80^{ts}. See Figure 4-1 B,

"GAL80^{ts} 18°C"). At 18°C, GAL80^{ts} successfully represses *dVMAT* expression and blocks

rescue of female sterility. Constitutively rescued flies reared at 18°C until shifted to 30°C at the

time of mating (beginning at 1 day post-eclosion) are fertile (90% of flies; Figure 4-1 B "rescue

18° → 30°C"). GAL80 rescue flies shifted under the same time frame are also fertile (95% of

flies; Figure 4-1 B "GAL80^{ts} 18°→30°C"). Together, these results suggest that VMAT

expression during development (from embryo to adult eclosion) is not necessary for rescue of

female fertility.

VMAT expression during behavioral testing not required for rescue of a VMAT-dependent adult
behavior

The GAL80^{ts} inducible system was also used to repress VMAT expression during adult

stages after expression during early development. Flies expressing ubiquitously driven VMAT

under control of the temperature-sensitive GAL80 repressor were reared at 30°C and then shifted

to 18°C as yellow pupa (Figure 4-1 C, smaller light brown triangle). Adult flies which eclosed

after this temperature shifting paradigm were sacrificed and VMAT protein expression was

measured in head homogenates by Western blot analysis. Flies show reduced VMAT expression

(Figure 4-1 C, "Δ yellow pupa") compared to flies raised continuously at 30°C (Figure 4-1 C,

"no Δ"); however, VMAT protein levels are still detected. When flies are reared at 30°C for a

shorter period of development and shifted to 18°C as 3rd instar larva, flies eclosing from this

temperature shift paradigm do not express detectable levels of VMAT protein expression as

adults (Figure 4-1 C, "Δ 3rd instar").

Female flies with the GAL80ts rescue genotype were shifted from 30°C to 18°C as either

brown pupa, yellow pupa, or 3rd instar larva were tested for fertility (Figure 4-1 D). Flies in all

three temperature shifting paradigms rescued sterility as well as flies which were reared

continuously at 30°C. This rescue is not seen in flies raised continuously at 18°C. These results

show that VMAT expression during the adult behavioral testing is not required for rescue of the

VMAT-dependent behavior.

A mutant allele of dVGAT is lethal

To investigate the function of *dVGAT in vivo*, we characterized a lethal allele of *dVGAT*

generated by the insertion of a *minos* transposable element within the second exon (METAXAKIS

et al. 2005). To identify the developmental phase at which *dVGATminos1* is lethal, I took

advantage of the fact that *minos* elements are marked with GFP (METAXAKIS *et al.* 2005). In

~200 embryos, I observed the expected Mendelian ratio of strongly labeled homozygotes, and

less intensely labeled *dVGATminos1*/*CyO* heterozygotes. None of the approximately 50 intensely

fluorescently labeled homozygous embryos were observed to hatch, confirming the *VGATminos1*

allele is embryonically lethal.

To help define the severity of the *dVGATminos1* allele, I probed for residual protein

expression using immunolabeling. I used the anti-dVGAT antibody followed by a fluorophore-

conjugated secondary antibody to label embryos derived from the *dVGATminos1*/*CyO* line (Fig.

4-2 A–D) and again took advantage of the GFP tag in the *minos* insertion. In homozygous

mutant embryos showing high levels of GFP expression (Fig. 4-2 A,B), we were unable to detect

any specific labeling for dVGAT (Fig.4-2 C,D). By contrast, in embryos showing intermediate

levels of GFP expression (Fig. 4-2 A,B), the ventral nerve cord was robustly labeled for dVGAT

(Fig.4-2 C,D). Higher resolution, confocal images of another heterozygote showing intermediate

levels of GFP expression (Fig. 4-2 E) confirm the specific labeling for dVGAT in the ventral

nerve cord (Fig. 4-2 F). There was no specific labeling for dVGAT in a homozygote showing

higher levels of GFP expression (Fig. 4-2 H–J). Autofluorescence of the gut in all embryos can

be seen in both the red (Fig. 4-2 F,I) and green channels (Fig. 4-2 E,H) as well as the merged

images (Fig.4-2 G,J). These data suggest that *dVGATminos1* is either a strong hypomorph or a

null allele, although we cannot rule out the possibility that it has some residual activity.

Western blots did provide sufficient sensitivity to quantify embryonic dVGAT

expression. dVGAT protein levels were below our limit of detection using up to 30 wild-type

embryos (Fig. 4-3), thus making it difficult to use western blotting to determine whether *dVGAT*

mutants might express residual dVGAT protein.

Inducible transgenes can rescue the dVGAT mutant thereby allowing behavioral analyses

To allow both constitutive and inducible rescue/knockdown of *dVGATminos1*, I used the

well-characterized GAL4/UAS system (BRAND and PERRIMON 1993). Spatially directed rescue

utilized *dVGAT-GAL4* and *UAS-VGAT* lines constructed by Julie Simpson. The GAL4 driver

lines contain 6.9kb upstream of the initiating methionine of *dVGAT*. In conjunction with the

UAS-VGAT transgene, the driver expresses the predicted 1.7kb open reading frame of *dVGAT* in

cells which are normally VGAT positive. *dVGATminos1* homozygotes containing *VGATGAL4*

(III) and *UAS-dVGAT(III)* (*dVGATminos1*; *dVGAT-GAL4(III),UAS-dVGAT(III)*) as well as

dVGAT mutants containing *dVGATGAL4(II)* and *UAS-dVGAT(III)* (*dVGATminos1*, *dVGAT-*

GAL4(II);UAS-dVGAT(III)) rescue the *dVGATminos1* allele and were both viable and fertile. In

addition, rescue using either *dVGAT-GAL4(II)* or *dVGAT-GAL4(III)* indicates that both GAL4

transgenes are expressed at least in those GABAergic neurons required for development and

survival.

Hao Fei and I found that transient expression of dVGAT during development rescues the

embryonic lethality of the *dVGATminos1* allele, and allow us to probe the adult mutant

phenotype and the contributions of GABA release to adult visual behavior. Wei Song Ong and I

constructed a line containing the homozygous *dVGATminos1* allele with *dVGAT-GAL4(II)*, *UAS-*

dVGAT(III) and a temperature-sensitive version of the GAL4 transcriptional repressor GAL80 on

chromosome III (MCGUIRE *et al.* 2003) (*dVGATminos1, dVGATGAL4 (II); UAS-dVGAT(III),*

tub-GAL80ts). The flies were cultured at 30°C, the permissive temperature for expression, until

they had completed embryonic and larval development. They were then shifted to 18°C at 50%

pupation to block further dVGAT expression.

Flies raised at 30°C then shifted to 18°C are referred to as dVGAT knockdowns. The

knockdown flies (Fig.4-4 A, K.D.) showed *dVGAT* expression levels ~10–20% of flies that were

maintained at 30°C and allowed to express dVGAT throughout development and adulthood (Fig.

4-4 A, 30°C Rescue). Constant exposure to 30°C limited the numbers of healthy flies (data not

shown) available for further behavioral experiments. Wei Song Ong and I therefore generated an

additional control line in which *dVGAT* was constitutively rescued (*dVGATminos1, dVGAT-*

GAL4(II); UASdVGAT(III)) regardless of temperature. Western blots showed that dVGAT

expression in the knockdown line (Fig. 4-4 A, K.D.) is ~10–20% that of the constitutively

rescued flies (Fig. 4-4 A, Const.Res.; differences in the relative intensity of the K.D. lanes in Fig. 4-4 A reflect differences in the exposure time for each blot).

To determine whether knockdown of *dVGAT* would cause gross neuroanatomical changes, I immunolabeled adult brains from both knockdown and control (constitutively rescued) flies, focusing primarily on the optic lobes. The neuropil of the medulla, lobula and lobula plate in control (Fig. 4-4 B) and knockdown (Fig.4-4 C) were labeled for CSP (ZINSMAIER *et al.* 1990) and appear similar if not indistinguishable. The lamina and proximal medulla from control (Fig.4-4 D) and knockdown (Fig. 4-4 E) animals are shown co-labeled with antibodies to the synaptic protein Bruchpilot (mAb nc82) (WAGH *et al.* 2006) and the neuronal marker Nervana (anti-HRP) (SUN and SALVATERRA 1995), the latter highlighting the projections of the photoreceptor cell axons into the medulla. Although we cannot rule out subtle changes in connectivity or synaptic structure, the anatomy of the lamina and the organization of the photoreceptor cell processes appeared to be intact in the *dVGAT* knockdown flies.

DISCUSSION

Inducible transgene expression systems provide useful tools for assessing the temporal requirement for protein expression. Vesicular neurotransmitter transporters act as gatekeepers for neurotransmitter release and temporal expression of these transporters act as useful probes for examining the circuits controlled by the vesicular neurotransmitter transporter. The experiments in this chapter use this control point to examine monoaminergic and GABAergic contributions to behaviors and elucidate the temporal requirements for each neurotransmitter type.

VGAT mutants provide an important model to investigate the contribution of GABA release to complex behavior. However, studies of VGAT mutants in other genetic systems have

been limited. The *C. elegans* VGAT mutant *unc-47* primarily disrupts peripheral GABAergic circuits that innervate the neuromuscular junction (SCHUSKE *et al.* 2004), and homozygous VGAT knockout mice die as embryos (WOJCIK *et al.* 2006). To allow the study of VGAT in the fly, we have developed a new antibody to *Drosophila* VGAT, generated GAL4 and UAS transgenic lines to express dVGAT, characterized a *dVGAT* mutant and constructed an inducible rescue line in which the *dVGAT* transgene is expressed during development but not adulthood.

Previous pharmacological studies have suggested that GABA may regulate optomotor behavior in the fly (BÜLTHOFF and BÜLTHOFF 1987; EGELHAAF and BORST 1993; WARZECHA *et al.* 1993), and we have used the *dVGAT* knockdown flies as a new genetic model to further test this hypothesis. Our behavioral results indicate that object detection is particularly sensitive to a decrease in GABA release and we discuss possible mechanisms below. *dVGAT* flies provide an important platform for dissecting the circuits underlying this phenotype, and more generally, allow further studies on the function of GABA release in a variety of other behaviors. We note that GAD, another marker commonly used to mark GABAergic cells is less specific than dVGAT, and is expressed in at least some glutamatergic cells (FEATHERSTONE *et al.* 2000).

The lethality of *dVGAT* larvae support the idea that dVGAT also plays an important role in development. To circumvent the early developmental requirements for *dVGAT*, we used a temperature-sensitive repressor of GAL4 (GAL80ts) to selectively knockdown expression in the adult. We constructed a line containing *dVGAT-GAL4, UAS-dVGAT* and a ubiquitously expressed *GAL80ts* transgene (*tub-GAL80ts*) in the mutant *dVGAT* background to generate the inducible knockdown line *dVGATminos1,dVGAT-GAL4(II); UAS-dVGAT(III), tub-GAL80ts*. The GAL80ts protein is functional at 18°C, thereby blocking the transcriptional activation of the *UAS-dVGAT* transgene by GAL4. Conversely, at 30°C, GAL80ts is non-functional and the *UAS-*

dVGAT transgene is activated. Thus, we generated an inducible knockdown line in which the embryonic lethality of *dVGAT* mutant could be rescued.

The survival of the *dVGAT* knockdown flies allowed us to test the effects of reduced dVGAT expression and GABA release on adult visual behavior. As controls for most studies, we used the constitutively rescued flies. Controls were cultured in parallel and carried through the same temperature shift (30° to 18°C at 50% pupation) as for the knockdown line.

I collaborated with Dawnis Chow, a graduate student in Mark Frye's lab, to assess general motor function and specific visual sub-systems in the dVGAT knockdown flies. Using a digital flight simulator, he found that dVGAT knockdown flies have intact general motion processing but disrupted visual figure detection.

Both control (constitutively rescued) and dVGAT knockdown groups were equally capable of stabilizing a wide-field panorama, which suggests that general motion processing and optomotor control circuits are intact despite reduced dVGAT expression. Flight control is simulated by coupling the fly's steering wing kinematics to the movement of the visual panorama. To test general wide-field optomotor control, we added a sinusoidal bias to the feedback controller to ensure that the flies were actively engaged in controlling the visual display rather than passively flying forward (Fig 4-5 A). Both groups also exhibited normal (wild-type) ranges in wing frequency and amplitude, indicating that the neural regulation and resultant mechanical power output of the flight motor system are intact in the mutant flies (GORDON and DICKINSON 2006).

However, knockdown flies showed a reduced ability to fixate a stripe moving against a uniform white background (Fig. 4-5 B). Knockdown flies also showed a diminished capacity for tracking a stripe moving against a counterrotating wide-field background in which any

displacement of the stripe was matched exactly by motion of the background in the opposite

direction (Fig. 4-5 C). Chow further investigated the temporal response properties by varying

the feedback dynamics of the object and the counter-rotating background under open-loop

feedback conditions in which the fly had no control over the display. We generated three stimuli:

one in which the object was phase-locked with the textured widefield background (i.e. 0 deg.

phase lag), one with a phase lags of 90 deg, and another with a 180 deg. Phase lag. We find that

the amplitude of behavioral responses by knockdown flies decreased progressively as the figure

and ground were moved further out of phase, whereas the phase of behavioral responses were

little impacted.

Together, these results suggest that general wide-field optomotor function is intact, and

the timing of visual signals is not significantly altered by *dVGAT* knockdown, whereas the

behavioral deficit in the *dVGAT* knockdown flies is specific for figure detection computations

required to follow or fixate the direction of gaze on a small-field object, and more so for the

control of response magnitude rather than timing.

Future studies can examine the specific GABAergic circuits responsible for disrupted

figure-ground detection in the dVGAT knockdown flies. Although it is known that monopolar

cells segregate visual information and contribute to early motion pre-processing (COOMBE and

HEISENBERG 1986; RISTER *et al.* 2007; ZHU *et al.* 2009), it remains controversial as to whether

motion computations are solely made within the deeper visual ganglia, the lobula (SINAKEVITCH

and STRAUSFELD 2004), and/or lobula plate (BROTZ and BORST 1996; BROTZ *et al.* 2001). CH

cells in the lobula plate have been shown to be involved in detection of moving objects against a

moving background (EGELHAAF and BORST 1993; WARZECHA *et al.* 1993). It is therefore

conceivable that the detection of all object movement is mediated by interactions between CH

and FD cells. Conversely, it is possible that some of the other ~1500 GABAergic cells in the optic ganglia contribute to the *dVGAT* phenotype. C2 neurons may be involved in orientation (DOUGLASS and STAUSFELD 1995) and both C2 and C3 provide input to monopolar neurons (TAKEMURA *et al.* 2008), which have been suggested to function as motion detectors (RISTER *et al.* 2007). *dVGAT* mutants and transgenes will facilitate further tests to determine how GABA release from C2, and other cells in the optic ganglia may contribute to motion detection. Our results complement these studies and allow us to assess, for the first time, the effects of decreasing the function of dVGAT and presynaptic GABA release on fly behavior.

Since dVGAT is expressed widely in the adult insect nervous system, the inducible VGAT expression used here to examine visual behavior can also be used to explore inhibitory input on other behaviors. In this study, my colleagues and I focused primarily on dVGAT expression in the optic ganglia and its possible contribution to motion detection; however, other behaviors can also be assessed using the approach delineated here. For example, multi-sensory integration may be modulated by GABAergic innervation. Local GABAergic interneurons adjacent to the antennal lobe are thought to regulate cross-talk between adjacent glomeruli and hone the fly's olfactory response to specific odors (OLSEN and WILSON 2008; SILBERING and GALIZIA 2007; WILSON and LAURENT 2005). dVGAT is also expressed in the central complex, consistent with reports using other GABAergic markers (HARRISON *et al.* 1996; HOMBERG *et al.* 1987; MEYER *et al.* 1986) may contribute to the role of GABA release in spatial working memory (NEUSER *et al.* 2008). My colleagues in the Krantz group also observed labeling for dVGAT in the calyx of the mushroom bodies, where GABAergic input may regulate Kenyon cell activity and olfactory learning (HOMBERG *et al.* 1987; LEITCH and LAURENT 1996; LIU *et al.* 2007; YASUYAMA *et al.* 2002). GABA has also be indicated to be involved with sleep regulation

(AGOSTO *et al.* 2008). We anticipate that *dVGAT* knockdown flies may be used to further explore the contribution of GABA release to these behaviors.

Flexibility in monoaminergic input requirements

In contrast to GABAergic requirements for expression during development for viability and figure-ground detection, monoaminergic circuits display a greater amount of flexibility in the temporal requirements for rescue of an adult behavior. Monoaminergic input provided either during early development or in later adult stages is sufficient to rescue female fertility, a behavior previously shown to be dependent on VMAT expression.

Vesicular neurotransmitter transporters display remarkable perdurance

dVGAT expression could be knocked down at later stages by shifting the flies from 30° to 18°C. Shifting the flies from 30° to 18°C during embryonic or larval stages did not yield any viable adults (data not shown) suggesting that *dVGAT* is required throughout larval development. We were surprised to find that shifting adult flies to 18°C for up to 1 week did not reduce dVGAT expression (data not shown). Other workers have been able to use GAL80ts to effectively reduce protein expression using as little as 1h of exposure to the restrictive temperature (MCGUIRE *et al.* 2003). It would appear that the dVGAT protein persisted despite the blockade of expression from the transgene. In other words, dVGAT showed a dramatic degree of perdurance.

The apparent perdurance of dVGAT might be due to limited recycling of the protein in the adult. To circumvent this problem, and still allow expression during development, we took advantage of the restructuring of the nervous system during metamorphosis. During pupal stages,

some of the larval nervous system is destroyed, and those aspects of the CNS used only in the adult develop from immature precursor cells. We speculate that by shifting to the restrictive temperature during pupation, we blocked *de novo* adult dVGAT expression before it could occur, thus circumventing problems with perdurance. Vesicular monoamine transporter (dVMAT) expression appears to be similarly resistant to knockdown as adults. Vesicular transporters are transmembrane proteins, and must be processed in the ER and Golgi in the cell body before trafficking to synaptic vesicles at the nerve terminal. Importantly, SVs undergo multiple rounds of recycling at the nerve terminal, and the processes by which SV proteins might be degraded or leave the nerve terminal are not known. Future experiments using vesicular transporter mutants defective in membrane trafficking may help address this question, and perhaps help to explain the perdurance of dVGAT and dVMAT.

FIGURES

Figure 4-1. Temperature-controlled dVMAT expression reveals developmental VMAT expression not required to rescue an adult behavior.

(A) GAL80^{ts} transgene regulates transcriptional activity in a temperature-dependent manner. Temperature does not affect dVMAT expression in constitutively rescued dVMAT flies, but does affect dVMAT expression in GAL80^{ts} mutant flies. Expression of UAS-dVMAT-A detected by probing with HA antibody which recognizes HA epitope inserted in first lumenal loop of dVMAT. Western blot of dVMAT^{P}/+; Daughterless-GAL4, UAS-dVMAT(III)/ tub-GAL80^{ts} (const) flies raised continuously at 18°C or shifted to 30°C 1 day post-eclosion express dVMAT and expression does not alter with temperature shifts. In contrast, dVMAT^{P}/ScO; Daughterless-GAL4, UAS-dVMAT(III)/ tub-GAL80^{ts} (Gal80ts) flies raised continuously at 18°C do not express dVMAT. VMAT expression appears after flies are shifted to 30°C 1 day post-eclosion (Gal80ts, 18° to 30°C). dVMAT expression in Gal80ts flies is stronger 3-days post-shift than 1-day post-shift (Compare "Gal80ts, 18° to 30°C, 1d" to "gal80ts, 18° to 30°C, 3d").

(B) VMAT expression during development is not required for rescue of a *dVMAT* adult behavior. When flies are raised continuously at 18°C, all wild-type (Canton-S; +/+) female flies tested are fertile and all *dVMAT* mutants are infertile. Constitutively rescued flies ("rescue 18°C"; dVMAT^{P}/dVMAT^{P}; Daughterless-GAL4, UAS-dVMAT(III)/+) that remain at 18°C through development and adulthood were fertile nearly 32 percent of the time. At 18°C, gal80ts blocks transcription of VMAT, and dVMAT^{P}/dVMAT^{P}; Daughterless-GAL4, UAS-

dVMAT(III)/Tub-Gal80^{ts} (gal80ts) flies remain sterile. When constitutive rescue and Gal80ts rescue flies are raised at 18°C and shifted to 30°C 1-day post-eclosion, females are fertile nearly 90 and 95 percent of the time, respectively.

(C) 30 → 18°C shift during early development can turn off VMAT protein expression. Flies were shifted during the stage indicated and adults were sacrificed 0-8 days post-eclosion. Head homogenates were probed for VMAT using an HA antibody that recognizes an HA-tag on VMAT.

(D) dVMAT^{P}/dVMAT^{P}; Daughterless-GAL4, UAS-dVMAT(III)/Tub-Gal80^{ts} (gal80ts) flies raised continuously at 18°C are infertile. Gal80ts flies which remain at 30°C through development and adulthood were fertile 58 percent of the time (n=43). When flies are shifted to 18°C at progressively earlier points in development, female flies remain fertile. Flies shifted at the brown pupal stage (56%, n=18), yellow pupal stage (80%, n=5), and third instar larval stage (100%, n=3) are fertile.
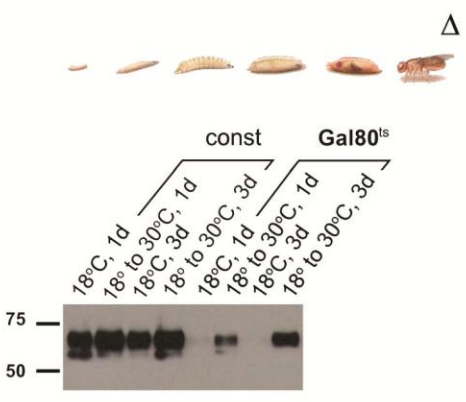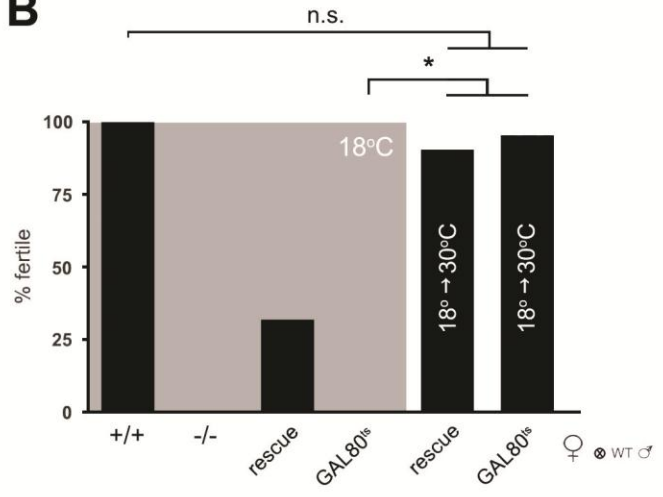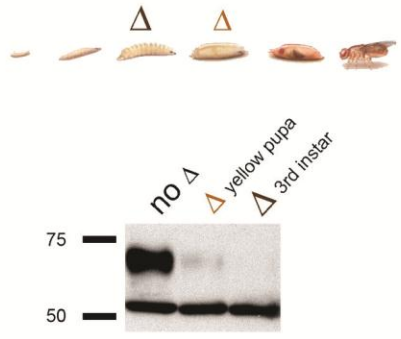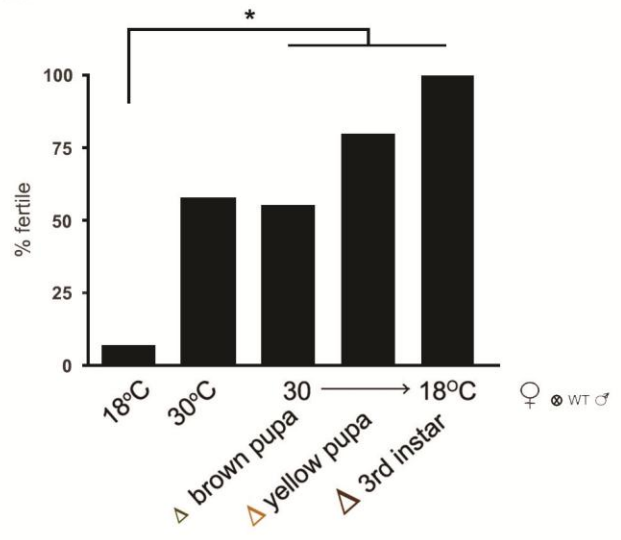
A

B

C

D

Figure 4-2. dVGAT mutant does not express dVGAT.

The *minos* insertion line CG8354^{MB01219} is GFP-tagged and homozygous and heterozygous

mutants can be identified by the GFP intensity. Here, embryos were derived from

dVGAT^{minos1}/CyO parents, and visualized using a standard upright (A–D) or confocal (E–J)

microscope. Embryos from two separate low-power fields are shown in A,C and B,D. For all

panels, anti-dVGAT, labeling is in red and endogenous fluorescence of GFP is green.

Intense GFP fluorescence (A,B white arrowheads) is seen in embryos homozygous for the GFP-

tagged minos insertion dVGAT^{minos1}/dVGAT^{minos1}. These embryos do not show specific labeling

for dVGAT in the ventral nerve cord (C, D, white arrowheads). Embryos showing less intense

GFP fluorescence (A,B, white arrows) are heterozygous for the mutation (dVGATminos1/CyO)

and show robust labeling for dVGAT in the nerve cord (C,D, white arrows). The remaining,

malformed embryo shown in B and D (asterisk) is presumably CyO/CyO. (E–G) Confocal

images of additional embryos. A dVGAT$_{minos1}$/CyO heterozygote (E–G) shows moderate GFP

fluorescence (E, white arrow) and labeling of dVGAT in the ventral nerve cord (F, white arrow).

A VGAT$_{minos1}$/dVGAT$_{minos1}$ homozygote shows high levels of GFP fluorescence (H, arrowhead)

and no specific labeling of dVGAT in the nerve cord (I, white arrowhead). As seen in the merged

images (G, J), both heterozygotes and homozygotes show nonspecific autofluorescence in the gut

(small grey arrowheads). Scale bars, 100 μm.

$$\frac{dVGAT^{minos1}}{CyO}$$

$$\frac{dVGAT^{minos1}}{dVGAT^{minos1}}$$

$$\frac{dVGAT}{dVGAT}^{minos1}_{minos1}$$

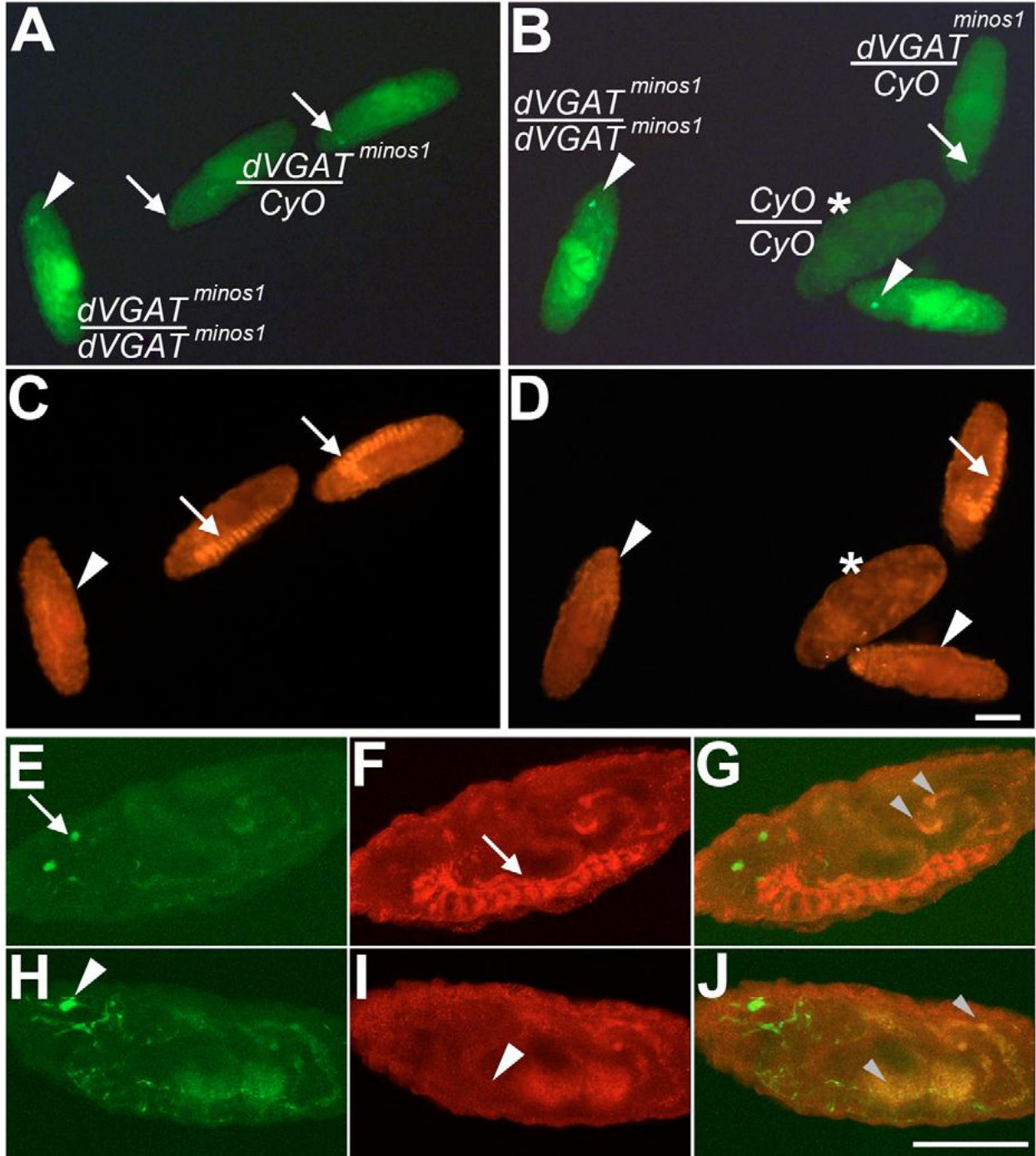$$\frac{dVGAT}{CyO}^{minos1}$$

$$\frac{CyO}{CyO} *$$

Figure 4-3. Embryonic dVGAT expression undetected by Western blot analysis.

Wild-type (Canton-S) embryos were dechlorinated, devitelinized and homogenized in the quantities denoted. Western blots probed with anti-dVGAT were unable to detect dVGAT in embryos though dVGAT is clearly detected in one adult fly head.
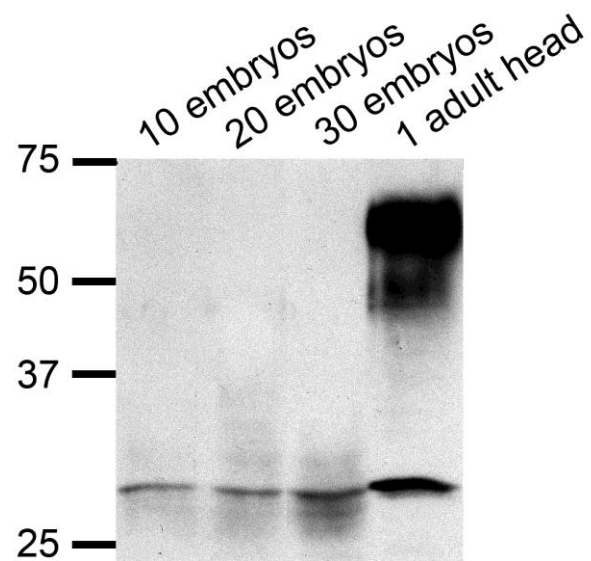
Figure 4-4. dVMAT knocked down in GAL80^{ts} mutant flies, but gross brain anatomy remains intact.

(A) Temperature controls dVGAT expression in GAL80^{ts} mutant flies. Western blots of

dVGAT$_{minos1}$, dVGAT-GAL4; UAS-dVGAT(III), tub-GAL80^{ts} flies raised continuously at 30°C

(30° Rescue) show high expression of dVGAT relative to flies shifted to 18°C at 50% pupation

and then maintained at 18°C (K.D.; knockdown). Wild-type flies (wt; CS strain) raised at 25°C

show an intermediate level of expression. In a separate experiment dVGAT$_{minos1}$, dVGAT-

GAL4; UAS-dVGAT (III) (constitutive rescue; Const. Res.) and dVGATminos1, dVGATGAL4;

UAS-dVGAT(III), tub-GAL80^{ts} flies (K.D.) were shifted in parallel from 30° to 18°C at 50%

pupation. The knockdown flies had lower levels of expression than the constitutively rescued

flies.

(B-E) No gross anatomical differences found between constitutively rescued and knockdown

flies. (B,C) Labeling for CSP in adult heads from constitutively rescued flies (F) cannot be

distinguished from labeling in knockdown (C) flies. (D,E) Co-labeling using mAb nc82 (red) and

anti-HRP (green) of adult heads from constitutively rescued controls (D) and knockdown (E)

animals are indistinguishable. Labeled structures in B-E include the medulla (M), lobula (Lo),

lobula plate (LP), lamina (L), and photoreceptor cell processes in the medulla (arrowheads).

Scale bars, (B-E) 50 um.
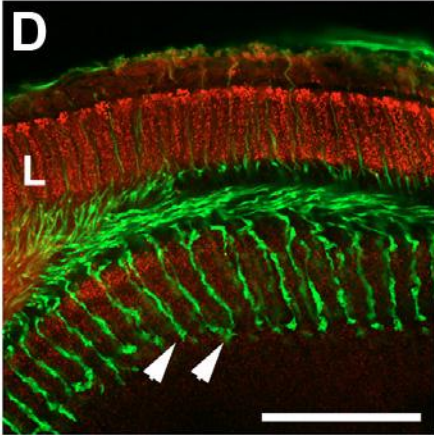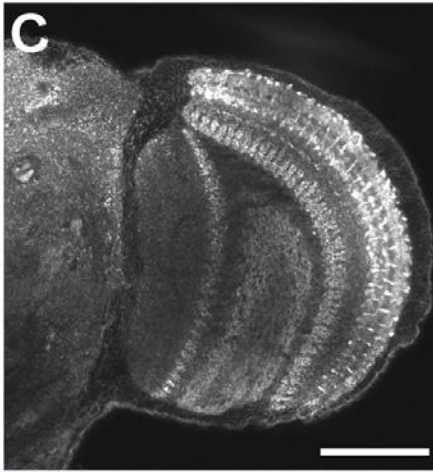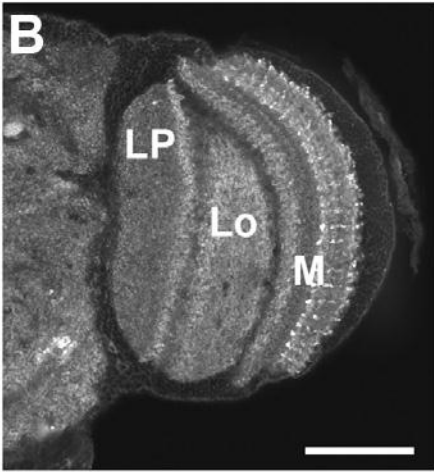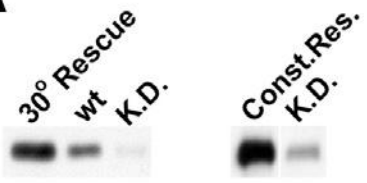
Figure 4-5. Visual stimuli used to test figure-ground detection.

(A) To test wide-field vision, flies had closed-loop control biased with a sinusoid over a wide-field high-contrast random checkerboard background panorama

(B) To test small-field object tracking, the display was switched to a uniform white panorama with a single dark vertical stripe located by convention at the 0-degree position, and flies were tested under biased closed-loop conditions.

(C) To test figure-ground detection, the small-field object (stripe) was coupled to the motion of the checkerboard background such that a clockwise movement of the object resulted in a counter-clockwise rotation of the background and visa versa. In initial closed-loop behavioral tests, the two stimuli were 180 deg. out of phase. In later open-loop conditions, in which the fly had no control over the display, the visual stimuli were either 180 deg. out of phase, 90 deg. phase lagged, or in phase (0 deg. out of phase).
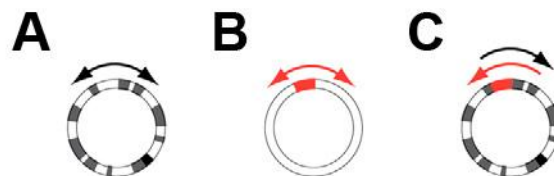
CHAPTER FIVE:

General Discussion

This dissertation examined mechanisms which regulate communication between monoaminergic neurons at the circuit level and at the synapse.

Exploring regulation further at the synapse

In order to understand the cellular mechanisms which regulate monoamine release, my work used fast-capture, real-time imaging of pH-sensitive pHluorinss to examine the trafficking of vesicular monoamine transporters (VMATs) *in vivo*. Since these transporters regulate the loading and storage of monoaminergic transmitters in secretory vesicles, differences in trafficking to release sites and to synaptic vesicles influence the strength of communication. Data presented in Chapter 2 suggest that the dileucine motif or another signal within the DVMAT C-terminus regulates transporter trafficking to the axon terminal, localization to synaptic vesicles, and endocytosis speed in a manner separate from the previously described tyrosine-based motif (GRYGORUK *et al.* 2010b).

Differential interactions with the endocytic machinery

Future studies may examine whether Y600A, the dileucine motif and other signals employ the same endocytic machinery. Potential differences may be revealed by co-expressing pHluorin-tagged wt and DVMAT trafficking variants with additional mutations in the genes encoding specific elements of the endocytic machinery. Multiple mutations in endocytic proteins have been previously characterized and could be combined with DVMAT-pHluorin transgenes using standard *Drosophila* genetic techniques. These include subunits of AP-2 and AP-3 as well as synaptojanin, and endophillin (DICKMAN *et al.* 2005; KOH *et al.* 2004; POSKANZER *et al.* 2003; VOGLMAIER *et al.* 2006; ZHANG *et al.* 1998).

AP-2 and AP-3 are thought to be involved in two distinct endocytic (or perhaps post-endocytic) pathways, with AP-2 mediating a faster, clathrin-dependent route, and AP-3 mediating a slower pathway involving an endosomal intermediate used for compensatory endocytosis (VOGLMAIER *et al.* 2006). Consistent with this, the tyrosine-based motif (YXXØ, where X is any amino acid and Ø is a bulky hydrophobic residue) in dVMAT is thought to interact with the μ subunit of AP-2 (EVANS and OWEN 2002; OHNO *et al.* 1995; ROBINSON 2004), and the motif has been shown to be critical for the rapid internalization of proteins from the plasma membrane (BONIFACINO and TRAUB 2003). It is possible that DVMAT trafficking mutants could be diverted from one endocytic pathway to another. Indeed, AP-3 coated vesicles are thought to deliver their cargo to lysosomes or late endosomes (ROBINSON 2004), and unpublished data from our lab has found that dVMAT in Δ3 trafficking mutants are mislocalized to lysosomes or late endosomes. In the absence of the dileucine motif in the Δ3 region, I hypothesize that the transporters lose an active signal to be directed to the faster, AP-2-mediated, clathrin-dependent pathway and the majority of dVMAT is rerouted to the AP-3 associated pathway. Experiments on mammalian VGLUT1 provide an important precedent for this hypothesis (VOGLMAIER *et al.* 2006): VGLUT1 variants which lack a second polyproline motif exhibit retarded endocytosis during prolonged stimulation by a mechanism that is brefeldin A (BFA)-sensitive, suggesting that the slower endocytosis process is mediated by AP-3. In contrast, VGLUT1 variants which lack both the second polyproline motif and the dileucine motif display endocytosis kinetics similar to wild-type, suggesting an alternate pathway for endocytosis, posited to be mediated by AP-2, since dileucine-like motifs can recruit AP2 for clathrin-dependent endocytosis. If AP3 differentially interacts with DVMAT trafficking mutants, both the *Drosophila garnet* mutant flies, which contain a mutation in the δ subunit of

AP3, and *ruby* mutants, which contain a mutation in the β subunit, will be differentially affected by the Δ3 and by Y600A VMAT mutations (ENG OOI *et al.* 1997; KRETZSCHMAR *et al.* 2000). Moreover, I project that Δ3 trafficking mutants will be unable to endocytose vesicles into the recently described "readily retrievable pool" of synaptic vesicles (HUA *et al.* 2011) and instead endocytose vesicles to an independent pool. Furthermore, double mutants which mutate both the dileucine and tyrosine motifs (DiLeu+Y600A) can be compared to single mutants to examine the complementary pathways available and identify the default pathway.

Another set of potentially interesting experiments would exploit previously characterized mutations in Drosophila synaptojanin (CREMONA *et al.* 1999) and endophilin (VERSTREKEN *et al.* 2002). Synaptojanin is a polyphosphoinositide phosphatase that converts phosphatidylinositol 4,5-biphosphate (PIP2) to phosphatidylinositol (WENK and DE CAMILLI 2004). Mouse mutants have an accumulation of clathrin-coated vesicles at nerve terminals (CREMONA *et al.* 1999), but studies of the *C. elegans* ortholog of synaptojanin, *unc-26*, suggest that the protein plays a role in *accelerating* steps within the synaptic vesicle recycling pathway rather than playing an essential role in recycling itself (HARRIS *et al.* 2000). It is possible that the Δ3 mutation in VMAT disrupts the transporter's ability to interact with synaptojanin, resulting in the observed slowed endocytosis dynamics.

Endophilin has also been shown to play a role in accelerating endocytosis, and the retarded endocytosis of Δ3 VMAT may reflect a disturbed interaction with endophilin. Past studies have shown that depletion of endophilin using anti-endophilin antibodies prevents formation of dynamin-coated buds on synaptic membranes in the lamprey spinal cord and in rat brain homogenates (RINGSTAD *et al.* 1999). In mammalian models, the SH3 domain of endophilin is thought to bind the second polyproline motif in the C-terminal cytoplasmic domain

of VGLUT1, whereby the interaction aids in efficient endocytosis after prolonged stimulation (VOGLMAIER *et al.* 2006). Similarly, boutons in the *Drosophila* endophilin mutant are depleted of vesicles (VERSTREKEN *et al.* 2002). In place of vesicles, mutants display shallow pits and no uptake of the lipid-dye FM 1-43. Based on these findings, Verstreken et al. suggest a kiss-and-run mechanism of release and endocytosis in these mutants. Controversial (DICKMAN *et al.* 2005), these observations nevertheless suggest that endophilin plays a pivotal role in endocytosis kinetics and argue for examination of how the Y600A and Δ3 mutations may be differentially affected by endophilin, whether as a direct or indirect interaction. There is not an obvious polyproline motif in DVMAT and it is therefore unlikely that we will observe precisely the same effects as seen for VGLUT. It is nonetheless possible that the endocytic complex containing endophilin and synaptojanin interacts with DVMAT and perhaps more specifically with one particular trafficking signal such as Y600A or other trafficking signals in DVMAT.

The trafficking mutants described in this dissertation may interact with the endocytoic machinery differently, but the candidates mentioned here are not exclusive of one another. Endophilin recruits synaptojanin during the uncoating process (SCHUSKE *et al.* 2003; VERSTREKEN *et al.* 2003), and endophilin relies on a pathway requiring AP-2 or AP-3 (VOGLMAIER *et al.* 2006). *In vitro* pull-down experiments further show a direct interaction between *Drosophila* synaptojanin and endophilin and Dap160 (VERSTREKEN *et al.* 2003). Although not well supported, it has been suggested that synaptojanin is also involved in the removal of AP-2 from the membrane (ROBINSON 2004).

VMAT localization to release-competent vesicles

Data presented in this dissertation also show that fewer mutant transporters are recruited to the plasma membrane during evoked release when motifs in the C-terminus of VMAT are mutated. My data suggest that fewer mutant transporters are localized to synaptic vesicles and fewer transporters are trafficked to the axon terminal for release. The combined effect of fewer transporters to release-competent vesicles and fewer transporters to release sites likely accounts for the decreased pHluorin response to electrical stimuli. To exclude the possibility that premature or excessive endocytosis is responsible for the smaller measurement of pHluorin signal during triggered exocytosis, future studies will examine evoked exocytosis during bath application of bafilomycin. Bafilomycin A1 inhibits vacuolar-type proton-ATPases, and bath application blocks reacidification of recently endocytosed vesicles (SANKARANARAYANAN and RYAN 2001). This measurement allows quantification of the cumulative number of transporters which fuse to the plasma membrane during exocytosis (KIM *et al.* 2009; KUROMI and KIDOKORO 2000; POSKANZER and DAVIS 2004; POSKANZER *et al.* 2003). Although low doses of bafilomycin have been shown to fragment early endosomes in murine macrophage cells (CLAGUE *et al.* 1994; DUCLOS *et al.* 2003) and have the potential to alter synaptic vesicle recycling involving endosomal intermediates (RIZZOLI *et al.* 2006), bafilomycin does not alter endocytosis, vesicle recycling or stimulus-induced calcium fluxes (SANKARANARAYANAN and RYAN 2001). Vesicles exocytose and endocytose normally under bafilomycin application, but vesicle lumens remain alkalinized after exocytosis. Therefore, pHluorin-tagged transporters increase in fluorescence intensity during evoked stimulation and intensity remains elevated during endocytosis, since budding vesicles are unable to reacidify. Simultaneous bath application of bafilomycin and electrical stimulation allows visualization of the total number of transporters that reside on vesicles that fuse to the plasma membrane during the stimulation

paradigm. When normalized to the amount localized to the nerve terminal, a decrease in the total number of transporters on fusion-competent vesicles in the Y600A or Δ3 mutants indicates ectopic localization to synaptic sites that are not release-competent. These release-incompetent sites could be extrasynaptic sites on the plasma membrane, intermediate organelles in the regulated secretory pathway, or a yet-to-be-identified synaptic vesicle pool with more stringent tethering and release mechanisms (SCHWEIZER and RYAN 2006; THOMSON 2000).

The number of transporters on release-competent vesicles can also be measured by co-expressing pHluorin-tagged VMAT with a mutation that affects the *shibere* locus, which encodes dynamin. The shibire[ts1] (shi) mutation blocks all synaptic endocytosis at the restrictive temperature, thereby retaining vesicle membrane components, such as VMAT, at the plasma membrane (CHEN *et al.* 1991; KOENIG and IKEDA 1989; VAN DER BLIEK and MEYEROWITZ 1991). Useful for measuring the cumulative number of VMAT recruited to the plasma membrane during evoked stimulus, this mutation forces recruited pHluorin-tagged VMAT to remain exposed to the alkaline extracellular space. In contrast to bafilomycin experiments, the *shi* experiments alter endocytosis and allow measurement of the total number of vesicles available for recruitment during evoked release by aberrantly retaining transporters at the plasma membrane after exocytosis (GRYGORUK *et al.* 2010b). Along with bafilomycin experiments, these experiments measure the total number of vesicles available for recruitment during evoked release.

In addition to measuring the total number of transporters expressed on released vesicles during evoked release, it is important to measure the number of transporters that reside on the plasma membrane at baseline. Surface pHluorin can be measured by measuring the pHluorin change following an acidic challenge. Past studies have successfully replacing HEPES with 2-

[*N*-morpholino]ethane sulphonic (MES) acid (pK = 6.1) to create a membrane-impermeable acid with a final pH of 5.5 to acid challenges (POSKANZER *et al.* 2003; SANKARANARAYANAN *et al.* 2000). Quenching of surface fluorescence is rapid (ATLURI and RYAN 2006), thus a 5-s MES quench can show the percentage of resting fluorescence that is attributed to surface dVMAT-pHluorin.

Trafficking signals for somatodendritic neurotransmitter release

During synaptic vesicle biogenesis, synaptic proteins exit the *trans* Golgi network (TGN) and constitutive secretory vesicles develop into mature synaptic vesicles after an endocytic step (FEI *et al.* 2008). It is presumed that after exiting the somatically located TGN, constitutive vesicles translocate down the axon and to release sites at the axon terminal. However, somatodendritic release is a known phenomenon (BUNIN and WIGHTMAN 1998; LI *et al.* 2005; NIRENBERG *et al.* 1996); thus, neuronal polarity signals are needed to direct synaptic vesicles either to somatodendritic sites or axonal sites. This may either take the form as a signal early in the secretory pathway that directs a constitutive vesicle to the soma or axon, or a signal later in the secretory pathway that tells a vesicle to return to the soma after maturation at the axon terminal (HORTON and EHLERS 2003). The data presented in Chapter 2 suggest that a signaling motif resides in the Δ3 region of VMAT which direct transporter localization to somatodendritic locations or to the axon terminal, in addition to signaling localization to synaptic vesicles and setting endocytic rates.

It is possible that both selective delivery and selective retention mechanisms are responsible for localization of VMAT at the axon terminal (HORTON and EHLERS 2003). In order to test whether Δ3 VMAT is unable to traffic to the axon terminal or whether it lacks a

signal to be appropriately retained at the terminal, future experiments can track Δ3 VMAT in the

neuron using fluorescence recovery after photobleaching (FRAP) (LOPEZ *et al.* 1988; PHAIR and

MISTELI 2001; REITS and NEEFJES 2001) or manipulations with a photoconvertible fluorophore

(ANDO *et al.* 2002; CHUDAKOV *et al.* 2004; GURSKAYA *et al.* 2006). Using pinhole shields to

limit areas exposed to laser photobleaching, specific regions in the soma, axon shaft or axon

terminal can be photobleached, and the neuron can be monitored for patterns in VMAT

trafficking in response. Fused to VMAT trafficking mutant variants, photoconvertible

fluorophores, such as Dendra, offer a gentler alternative to monitoring VMAT protein dynamics

(GURSKAYA *et al.* 2006; SHANER *et al.* 2007). Dendra-tagged VMAT exposed to a brief pulse of

488nm light are converted from green to red, while newly synthesized VMAT remain green.

Although other photoconvertible fluorophores are brighter and more photostable than Dendra,

Dendra is currently the only monomeric variant and less likely to sterically hinder VMAT

interactions with the endocytic pathway or vesicle budding processes (SHANER *et al.* 2007).

Experiments which examine VMAT traffic out of the soma and retrograde traffic away from the

axon terminal will elucidate whether VMAT Δ3 lacks an active signal to direct it to the axon

terminal or whether it lacks a retention signal to maintain VMAT localization at the terminal

after initial export from the soma. Photoconvertible fluorophores can also be used as a

"highlighter" to label a subpopulation of tagged VMAT and follow trafficking to potential

somatodendritic sites with a high signal to background ratio (REMINGTON 2006). These

experiments will allow us to test whether VMAT Δ3 lacks trafficking motifs to all release sites,

axonal and somatodendritic, or whether it simply lacks a signal to maintain expression at axonal

sites.

As we become aware of signals which dictate transporter movement to dendritic or somatic release sites, future studies may also examine the dynamics of exocytosis and endocytosis at these sites. These experiments will need to use a driver with less global expression than the pan-neuronal *elav-GAL4* in order to prevent over-saturated luminence at the cell-rich ventral ganglion. The *Tdc-GAL4* driver, previously described in this dissertation, labels 37-50 cell bodies in the larval ventral ganglion which sends projections to Type II neuromuscular junctions along with other contacts, and can be used to label a subset of the neurons in the ventral ganglion for these experiments (Chen et al., unpublished data; VOMEL and WEGENER 2008). Somadendritice release sites can be stimulated either by antidromic electrical stimulation through a suction electrode placed en passant on the axon or by high potassium stimulation, which depolarizes the cell to elicit an action potential. These experiments may reveal that VMAT trafficking variants may play unique roles at somatic dendritic locations relative to roles at the axon terminal.

Co-dependence of aminergic input: Layering temporal control with spatial specificity at the circuit level

By genetically dissecting the functions of individual or combinations of aminergic neurotransmitters in the regulation of *Drosophila* behavior, my work also found that certain *Drosophila* behaviors rely predominantly on a single aminergic system, although others require multiple aminergic systems that may function redundantly or in concert in the coordination of behavior. Future work may explore the temporal orchestration of monoaminergic input on coordinating behavior. Evidence presented here (Chapter 3) suggests that octopamine mediates the initial peak startle response while serotonin regulates the post-startle behavior, including

speed of acclimation and asymptotic velocity. It is possible that octopamine's role in regulating

startle behavior is temporally segregated from serotonin's role, such that octopamine is

dispensable at later stages of the startle behavior. In order to induce two separate expression

patterns that do not overlap in either temporal or spatial space, a chemically inducible LexPR

system, can be used in conjunction with a temperature-induced GAL4/UAS system

(EMELYANOV and PARINOV 2008; LAI and LEE 2006).

Efforts were also made to examine the temporal requirement for aminergic signaling

during development using inducible transgene expression systems available in the *Drosophila*.

Using these tools in the vesicular GABA transporter mutant, I successfully show that the

temperature-sensitive GAL80 system can be used to rescue inhibitory neurotransmission and

GABA release during development followed by knockdown in the adult, revealing roles for

GABA in figure-ground visual behavior. Future work investigating the temporal flexibility of

aminergic signaling may want to explore if transient amine release triggers long-term anatomical

reorganization or activation of downstream targets which bypass the need for either

developmental expression or adult expression of vesicular monoamine transporters. Studies

using inducible transgene expression systems to examine the temporal requirement for

neurotransmitter release will also benefit by finding ways to reduce the perdurance of vesicular

neurotransmitter transporter expression.

The studies presented in this dissertation examined signals used to target vesicular

monoamine transporters to synaptic vesicles and the spatial and temporal requirements for amine

signaling, but experiments presented here did not address environmental influences which

interact with genes in a gene-environment interaction (GxE) to influence behavior. Functional

polymorphisms in genes encoding enzymes involved in metabolizing monoaminergic

neurotransmitters, monoamine oxidase type A (MAO-A) and catechol-O-methyltransferase (COMT) and allelic variation in the serotonin reuptake transporter gene-linked polymorphic region (5HTTLPR) and tryptophan hydroxylase-related gene regions suggest that effects of genotype may be small (or undetectable) if variability in exposure to environmental adversity is not taken into consideration (CASPI *et al.* 2002; CASPI *et al.* 2005; CASPI *et al.* 2003; ELEY *et al.* 2004; HOSÁK 2007). Although several of these genes indicate an independent link to behaviors relevant to psychiatric disorders, most genes' connection to related behavioral phenotypes would have been negated in error without a GxE approach (MOFFITT *et al.* 2005).

Genes controlling complex behaviors, such as those relevant to psychiatric disorders, are likely to be pleiotropic, and behavioral phenotypes are likely to have multivariate bases. Genes involved in monoaminergic transmission have been implicated in psychiatric disorders, but conclusions have been incongruous and stress the need for basic science research in the field as we seek to understand regulation of transmission at monoaminergic neurons from the synapse up.

Experimental Procedures

Chapter Two: Probing Synaptic Vesicle Dynamics in a *Drosophila* Vesicular Monoamine Transporter (DVMAT) Trafficking Mutant

Construction of UAS-VMAT-pHluorins

Hao Fei engineered an XmaI(c|ccggg) site within the HA tag sequence of pMT-VMAT-A-HA (GREER *et al.* 2005) using Quikchange Site-Directed Mutagenesis Kit (Strategene). cDNA from the synthetic construct superecliptic pHluorin excised from a pBS-pHluorin plasmid by AgeI (a|ccggt) and ligated into the XmaI site to replace the HA tag with a pHluorin tag. The resulting pMT-VMAT-pHluorin was cut and inserted to pExp-UAS (Exelixis) via the 5'EcoRI and 3' XbaI sites, and pExp-UAS-VMAT-A-pHluorin was injected in the w^{1118} strain (BestGene Drosophila Embryo Injection Services). UAS-Y600A-VMAT-pHluorin and UAS-D3-VMAT-pHluorin transgenic flies were constructed in a similar fashion using VMAT mutation lines previously described in Grygoruk et al. (2010b).

Flies

Males from UAS-VMAT-pHluorin lines were crossed with female elav-GAL4 (on X), and heterozygous female progeny were used for pHluorin experiments.

Solutions

Third instar larvae were dissected in chilled Ca^{2+}-free HL3.1 saline (FENG *et al.* 2004) adjusted to pH 7.32. All experiments were performed in "recording solution": HL3.1 solution (pH 7.32) supplemented with 2.0 mM calcium (to allow normal rates of exo-endocytosis) and 7mM L-Glutamic Acid (to block muscle contraction for imaging). Stock solutions of monosodium-conjugated L-Glutamimic Acid (Sigma) were replenished on a weekly basis and imaging

solutions were made fresh daily. Ammonium chloride (NH_4Cl) HL3.1 saline was made by replacing 50mM NaCl with NH_4Cl and adjusting to pH 7.32.

Live imaging and analysis

Images were captured on a Zeiss Axio Examiner Z1 microscope with an Achroplan water-immersion objective (100x, 1.0 N.A.). Images were acquired with a cooled back-illuminated electron-multiplying CCD camera (Andor iXon3 897). Preparations were excited by a DG4 light source with a GFP Brightline® Filter Set (Semrocks) fitted for the Zeiss filter cube. Continuous time-lapse imaging (50 ms exposures) was made possible by adapting the Andor Piezo software to capture image bursts at one z-plane.

For data quantification, individual boutons from Type Ib NMJs in abdominal segment A4, muscle 13 were tracked during movement with the MeasureStack plugin in ImageJ. Boutons which moved out in or out of focus during image acquisition were discarded from analysis. Since individual boutons within Type Ib NMJs in an individual did not appear to vary significantly, all bouton data within one individual were averaged and counted as one trial. Traces are corrected for photobleaching, and dF/F is calculated as (Fpeak - Fbaseline) / Fbaseline, where Fpeak is the average of the 10 frames during the 0.5 s after stimulus offset, and Fbaseline is the average of the 10 frames (0.5s duration) prior to stimulus.

To calculate endocytosis rates, the dF/F data collected from the first 30 seconds after stimulus offset is fit to a one phase decay curve using a least squares (ordinary) fit using GraphPad Prism. A nonlinear regression is run without constraining the starting point (Y0) or plateau, so reports of tau reflect the rate of endocytosis without consideration of extent of endocytosis.

Alkaline-wash experiments

Synapses were stimulated for 2 seconds at 40 Hz in the HL3.1 recording solution described in the Solutions section and imaged for 1.5 minutes before the recording solution in the bath was replaced with ammonium chloride solution (pH-matched to recording solution). Fillets were incubated in NH_4Cl solution for 5 minutes and imaged. Measurements of intensity were taken from an average of five 50-ms exposure images taken sequentially immediately after the 5 minute incubation and used the same boutons selected for dF/F analysis.

Western blots

Larval brains from two female and two male third-instar larvae were dissected and homogenized in SDS-DTT sample buffer using glass on glass micro-tissue grinder (Kontes), then briefly microfuged. One-head equivalents from each genotype were loaded on a 10% polyacrylamide gel and blotted to nitrocellulose membranes. Samples were co-probed using a monoclonal antibody to GFP (1:1000; Invitrogen) and mouse anti-β-tubulin (1:4000; Accurate Chemical & Scientific Co.) followed by goat anti-mouse HRP-conjugated secondary antibody (1:2000; Biorad) and a chemiluminescent substrace (Pierce SuperSingal West Pico). To quantitate band intensity, films were scanned (Epson Perfection 2450), and integrals of band intensity were calculated using the ImageJ Single Panel Gel Analyzer Macro.

Static imaging

To capture and quantify fluorescent intensity of pHluorin-tagged VMATs at the cell body and at the nerve terminal across genotypes, endogenous fluorescence was measured in unfixed,

dissected ventral ganglia and fillet preparations. Dissected ventral ganglia were mounted in glycerol and placed under a glass coverslip in order to image all cell bodies in one Z-plane. Images were taken with a W N-Achroplan water-immersion objective (10x, 0.3 N.A.) by creating a water column between the objective and coverslip. Images of nerve terminals in HL3.1 (pH 7.32) were taken using an Achroplan water-immersion objective (100x, 1.0 N.A.). For each individual, 200 images were taken over 10s and averaged to create the composite snapshot. All images of ventral ganglion fluorescence were taken without electron multiplying gain to prevent pixel saturation.

Chapter Three: Selective Neuronal Restoration of Vesicular Monoamine Transporter Function Uncovers Coordinated, Complementary and Dispensable Roles for Dopamine, Octopamine and Serotonin in *Drosophila melanogaster*

Drosophila husbandry

For larval locomotion assays, the w^{1118} mutant outcrossed into Canton-S for 10 generations ($w^{1118}CS_{10}$) was used as the wild type (wt) control, and for rhythmicity assays, the w mutant was used as the wt control. For all other experiments wt controls were Canton-S (CS). The *dVMAT* null, homozygous for the loss of function allele (*dVMAT^{P1}*), and *UAS-DVMAT* transgene have been previously described (CHANG *et al.* 2006; OH *et al.* 2003; ROMERO-CALDERON *et al.* 2008; SIMON *et al.* 2009). Note that the *UAS-DVMAT* transgene used here encodes the neuronal isoform of DVMAT (DVMAT-A); a distinct RNA splice variant of *dVMAT* (DVMAT-B) is expressed only in a small subset of glia in the visual system and unlikely to be relevant to the behaviors discussed here (GREER *et al.* 2005; ROMERO-CALDERON *et al.* 2008). Gal4 driver lines include those previously shown to drive expression in serotonergic (*TrH-Gal4*) (PARK *et al.* 2006), dopaminergic (*TH-Gal4*) (FRIGGI-GRELIN *et al.* 2003), tyraminergic & octopaminergic (*Tdc2-Gal4*) (COLE *et al.* 2005) and both serotonergic and dopaminergic (*Ddc-Gal4*) (LI *et al.* 2000) neurons. To reduce the effects of genetic background on behavior, all drivers, as well as the *dVMAT^{P1}* allele and the *UAS-DVMAT* transgene were outcrossed for 5 generations into wild type background ($w^{1118}CS_{10}$). The outcrossed transgenes were introduced into the *dVMAT^{P1}* mutant background to generate the following genotypes, used for the transgenic rescue experiments described in the text: (1) "ubiq^{-5HT}:" *w; dVMAT^{P1}; daughterless-Gal4, UAS-DVMAT / +, (2)* "Ddc:" *w; dVMAT^{P1}, DDC-Gal4; UAS-DVMAT,* (3) "Tdc:" *w; dVMAT^{P1}, Tdc2-Gal4; UAS-DVMAT,* (4) "TH:" *w; dVMAT^{P1}; TH-Gal4, UAS-DVMAT,* (5) "TrH:" *w; dVMAT^{P1}, TrH-*

Gal4; UAS-DVMAT, (6) "Tdc + TH:" *w; dVMAT^{P1} / Tdc2-GAL4, dVMAT^{P1}; UAS-DVMAT / TH-Gal4, UAS-DVMAT,* (7) "Tdc + TrH:" *w; dVMAT^{P1}, TrH-Gal4 / dVMAT^{P1}, Tdc2-Gal4; UAS-DVMAT, (8)* "TH + TrH:" *w; dVMAT^{P1} / TrH-GAL4, dVMAT^{P1}; UAS-DVMAT / TH-Gal4, UAS-DVMAT;* (9) "UAS:" *w; dVMAT^{P1}; UAS-DVMAT.* A single copy of the *daughterless-Gal4* and *UAS-DVMAT* was used to provide DVMAT expression levels more comparable to other drivers. All lines were maintained on standard agar-molasses based fly food made by the UCLA fly facility: 10.7 g/L agar, 28.6g/L yeast, 71 mL/L molasses, 71 g/L cornmeal, 5.7 mL/L propionic acid, and 16mL/L of a 10% g/mL solution of methyl-paraben used as an anti-fungal agent.

Western blots

Western blots were performed as previously described (CHANG *et al.* 2006). Briefly, flies were anesthetized using CO_2, and 4 heads per genotype homogenized in SDS-PAGE sample buffer. One head equivalent of homogenate from each fly line was loaded onto a polyacrylamide gel, followed by transfer to nitrocellulose. The membrane was incubated with 1:1000 mouse anti-HA (Covance Research Products, Denver, CO) overnight at 4 °C and then incubated with 1:1000 mouse anti-β-tubulin (Accurate Chemical and Scientific, Westbury, NY) for 1 hr at RT. Membranes were incubated in secondary antibody for 1 hr at room temperature using 1:1000 anti-mouse HRP conjugated antibodies (Amersham Biosciences, Piscataway, NJ). The protein bands were detected using SuperSignal West Pico Luminol/Peroxide (Thermo Scientific, Rockford, IL) and exposed for 15-60 seconds on Kodak (Rochester, NY) Biomax Light Film.

HPLC

HPLC analysis of dopamine and serotonin was performed as previously described (CHANG *et al.* 2006). For all experiments to measure dopamine and serotoninwe used four 2-3 day old female heads per sample. Fly heads were manually collected and homogenized in 0.1M perchloric acid containing 0.1% EDTA using a glass on glass micro-tissue grinder (Kontes). Insoluble debris was sedimented by centrifugation and the supernatant filtered through a 0.22 μm Millipore MC cartridge. To measure octopamine, brains were dissected from four 2-3 day old females for each sample and processed as above.

Viability

To obtain flies for viability assays, ten males and ten females heterozygous for the *dVMAT* mutation were mated for 3 days at ~23ºC in bottles containing standard fly food. For all genotypes, the percentage of progeny homozygous for *dVMAT^{P1}* was compared to the number of homozygous progeny predicted by standard Mendelian ratios. The number of F1 *dVMAT* homozygote and total progeny were summed over 19 days post-mating. Bottles containing 500-1000 progeny were scored as standard culture conditions for calculating percent survival in Figure 2G.

Anatomical analysis

To image dopaminergic neurons, wt and *dVMAT* mutant larvae were dissected and fixed in 4% paraformaldeheyde, then probed with Rabbit anti-DTH, a gift of W. Neckameyer (St. Louis University School of Medicine), followed by anti-Rabbit Alexaflour 488 (Invitrogen Molecular Probes, Oregon, USA). For serotonergic and octopaminergic neuron counts, one copy of *UAS-mCD8-GFP* and one copy of either *TrH-Gal4* or *Tdc2-Gal4* respectively were expressed either

in the wt or *dVMAT* mutant background., and the endogenous fluorescent signal of GFP was used to count cell bodies in larval brains fixed in 4% paraformaldehyde. All images were obtained using a Zeiss confocal microscope with a 20x Plan-Apochromat (0.75 N.A.) objective.

Larval locomotion

To obtain larva homozygous for the $dVMAT^{P1}$ in the presence or absence of additional rescue transgenes, 20 males and 20 females were mated for 1 day at ~23ºC in a 6cm plastic dish filled with 20 mL of standard molasses fly food. Approximately one week later, 3rd instar larvae were collected. To assay larval locomotion, a single third instar larva was placed on a 14.5 cm diameter plastic dish filled with standard fly food and allowed to acclimate for 1 min. The lid of the dish was covered with a 5 x 5 mm grid and the number of grid lines crossed by the larva was manually recorded over a period of 5 min. Experiments were performed blindly with respect to genotype. All larvae were then allowed to pupate and eclose as adults: individuals homozygous for the *dVMAT* mutation were confirmed post-hoc by the absence of the CyO chromosome. Only data collected from $dVMAT^{P1}$ homozygotes were used for further analysis.

Neurotransmitter feeding

To make plates for drug administration, molten fly food was mixed with red food dye (Kroger, Cincinnati, OH, 9.6% final concentration) and an aqueous stock solution of neurotransmitter for a final neurotransmitter concentration of 10mg/mL. Third instar larvae with colored abdomens were tested using the locomotion assay described above. Larvae were allowed to feed for 4hrs and were tested at the start of feeding, after 2 hrs of feeding, and at the end of feeding.

Fertility

To obtain adult flies for fertility assays, 10 males and 10 females heterozygous for the *dVMAT* mutation were mated for 3 days at ~25ºC. Homozygous progeny were sorted under cold-anesthesia over ice. To test male fertility, one male candidate (0-5 day old) was mated with 3 wt virgin females in a vial. To test female fertility, one virgin female candidate (0-4 day old) was mated with 3 Canton-S males in a vial. One to two weeks after initial mating, candidates were scored as either fertile or infertile based on whether the vial contained at least one progeny (larva, pupa or adult). Only vials containing at least one male and at least one female the day after initial mating were scored.

Negative geotaxis

Negative geotaxis assays were performed as described in Simon et al. (2009). Briefly, 2-5 day-old male and females were cold anesthetized and allowed to recover for 1 day prior to testing. For each trial, 20 flies were loaded into a choice-test apparatus and the percentage of flies which climbed to the upper tube in 15 sec after tapping the apparatus on the benchtop three times was recorded. At least 160 naïve flies were tested for each genotype.

Response to startle (puff-o-mat)

Males (2–4 days old) were CO_2 anesthetized and allowed to recover for 2 days prior to testing. Flies were reared on a 12 hr day-night cycle at 25ºC. Temperature for behavioral experiments was maintained at 23-25ºC. For each assay, ten flies were manually loaded into tubes and allowed to acclimatize for 10 min prior to filming. Activity was recorded beginning at 1 min before delivery of the puff stimuli, until 3.5 min after stimulus termination. Each air puff (35 psi)

lasted 200 ms with a 5 s inter-puff interval. Movies were analyzed using custom locomotor tracking software (described in Lebestky et al. 2009).

Courtship

To obtain courtship candidates, 20 males and 20 females heterozygous for the *dVMAT* mutation, or wt controls, were mated in bottles for 3 days at ~25ºC. Cold-anesthesia was used to collect homozygous male progeny, control males and virgin females. Flies were aged 3-7 days in vials with fly food and were passed into fresh vials both the night before and the morning of testing. Using very brief (approx. 10 seconds) cold-anesthesia, a single male was paired with a single wt virgin female in a polypropylene chamber (8 mm inner diameter x 4 mm height) and digitally recorded for a maximum of 30 minutes or until copulation occurred. All courtship assays were performed in a dedicated test area maintained at ~23°C and ~80% humidity. Male following of the female and wing song were scored as male courtship behaviors. Courtship index (CI) was calculated as the total time in which the male spent performing courtship behaviors as a percentage of total observation time prior to copulation or as a percentage of 30 minutes if copulation never occurred within the designated 30 minute time period.

Circadian behavior

Behavioral data were collected with the Trikinetics Drosophila Activity Monitor (DAM) system. One to three day old flies were loaded in DAM monitors and activity data was collected for 10-12 days at a constant temperature of 23°C inside an enclosed incubator. During each experiment, flies were first maintained under an LD 12:12 schedule for 3-4 days to collect entrainment data, followed by a constant dark period (DD) for 7-8 days. The signal processing toolbox algorithms

(LEVINE *et al.* 2002) within MATLAB (MathWorks) were used to estimate circadian periods and to visualize actograms of individual flies. Differences in rhythmicity among genotypes were determined using the Rhythmicity Index (RI, a measure of robustness), the correlogram (a statistical measure of rhythmicity), and the pattern of activity (as assessed by examining actograms). Activity under LD conditions was scored as bimodal if both the morning and evening activity peaks were at least two-fold higher than the baseline (mean) activity in the actograms. Flies were scored as entrained if they satisfied the following 3 criteria under LD conditions 1) bimodality in each 24-h period 2) statistical significance for the correlogram, 3) an R.I. value >0.1.

Behavior under DD conditions (Table 2) was scored for those flies that were entrained under LD conditions and still alive after 5 days of constant darkness conditions. To increase the sample size under DD conditions for the TH, wt and *dVMAT* mutant, additional flies not tallied in Table 1 were tested and included in the tally for Table 2 and the analysis of rhythmicity under DD conditions. The percentage of flies that were rhythmic (% rhythmic) under DD conditions was calculated as NR/NB, where NR is the total number of rhythmic flies and NB is the total number of flies with bimodal behavior (during LD) that survived for at least 5 days of DD. For both LD and DD conditions, R.I. values were assessed using the Kruskal-Wallis test (non-parametric ANOVA) with Dunn's Multiple comparisons test (InStat 3, GraphPad).

Chapter Four: Temporally Restricted Requirements of Vesicular Neurotransmitter Transporters Differ in Aminergic Systems and GABAergic systems

Fly husbandry

Drosophila were cultured on standard cornmeal medium at 25°C except as noted below. The following fly lines were obtained from the Bloomington *Drosophila* Stock Center at Indiana University (Bloomington, IN, USA): *tub-GAL80ts* (stock 7018) and the *minos* insertion line *CG8394MB01219* (designated in the text as *dVGATminos1*), maintained over the *CyO* balancer chromosome. *dVGATminos1* and *dVMAT^P* were outcrossed for five generations in the wild-type strain *w1118* (Canton S background).

To obtain *dVGAT* knockdown animals for behavioral experiments, flies were allowed to mate and lay eggs in vials containing standard food at 30°C for up to 5 days, and the vials observed twice daily for evidence of pupation. Under these conditions, the time from the beginning of pupation to eclosion was ~80 h, and flies were moved to 18°C at ~40 h or 50% completion. Flies that eclosed the day after transfer to 18°C were discarded, and those that eclosed on subsequent days were collected and aged for 5–7 days at 18°C. Female flies were used for all dVGAT behavioral experiments.

For dVMAT rescue experiments, the previously described *UAS-VMAT-A* transgene expressed under the control of the ubiquitously expressed *daughterless-GAL4* driver (SIMON *et al.* 2009) was used as a constitutive rescue line. For inducible rescue experiments in *dVMAT* flies, tub-GAL80ts was introduced, and w; *dVMAT*; daughterless-GAL4, UAS-dVMAT-A/tub-GAL80ts flies were tested and compared to the constitutive rescue line. For experiments in which rescue expression was restricted to adult stages, flies were switched to 30 degrees at the time of mating.

Western blot

Western blots for dVMAT expression were performed as previously described for Chapter 3.

To probe for dVGAT epression, embryos or adult heads of the denoted quantities were homogenized in SDS-PAGE sample buffer. Samples were loaded onto a 10% polyacrylamide gel followed by transfer to nitrocellulose membranes. The membrane was incubated with anti-dVGAT (1:250) overnight at 4°C and then incubated with1:1000 anti-rabbit HRP conjugated antibody. The protein bands were detected using SuperSignal West Pico Luminol/Peroxide (Thermo Scientified, Rockford, IL).

Immunolabeling

Embryos were aged for 15–18h, dechorinated in 50% bleach, then devitellinized and fixed in 50% heptane, 2% paraformaldehyde, PBS for 40 min at ambient temperature, followed by washes in ethanol and PBS containing 0.2% Triton X-100 (PBST). Embryos were incubated for 30 min at ambient temperature in PBST containing 10% fetal bovine serum and in anti-dVGAT (1:200) overnight at 4°C. Secondary antibody (anti-rabbit Alexa Fluor 555; Molecular Probes/Invitrogen, Eugene, OR, USA) was used at 1:1000, with an overnight incubation at 4°C to improve permeabilization. After washing, embryos were mounted on coverslips using Aqua Poly/Mount (Polysciences, Warrington, PA, USA).

For immunolabelings of adult fly brains, brains were dissected in PBS and fixed in 4% paraformaldehyde (in PBS) for 2h at ambient temperature. Brains were then washed in PBST and blocked for 1 h at ambient temperature in PBST containing 5% fetal bovine serum. Primary anti- *Drosophila* Cysteine string protein (mouse mAb 1G12; Developmental Studies Hybridoma

Bank, University of Iowa) was diluted 1:25 in PBST and incubated overnight at 4°C. Additional primary antibodies included 1:125 rabbit antihorseradish peroxidase (HRP; Sigma-Aldrich, St Louis, MO, USA) and 1:10 mouse anti-Bruchpilot (mAb nc82; Developmental Studies Hybridoma Bank, University of Iowa). Secondary antibodies included 1:2000 dilutions of one or more of the following: anti-rabbit Alexa Fluor 488 or 555 and anti-mouse Alexa Fluor 488 or 555. After washing, brains were equilibrated in 70% glycerol in PBS and mounted on coverslips using ProLong Antifade (Molecular Probes/Invitrogen, Eugene, OR, USA).

Fertility

Fertility was measured as binary value as either fertile or not fertile. See materials and methods from Chapter 3 for details on this behavioral assay.

BIBLIOGRAPHY

ABDALLAH, L., S. J. BONASERA, F. W. HOPF, L. O'DELL, M. GIORGETTI *et al.*, 2009 Impact of Serotonin 2C Receptor Null Mutation on Physiology and Behavior Associate with Nigrostriatal Dopamine Pathway Function. Journal of Neuroscience 29: 8156-8165.

ADAMO, S. A., 2008 Norepinephrine and octopamine: linking stress and immune function across phyla. Invertebrate Survival Journal 5: 12-19.

ADAMO, S. A., C. E. LINN and R. R. HOY, 1995 The role of neurohormonal octopamine during 'fight or flight' behaviour in the field cricket Gryllus bimaculatus. Journal of Experimental Biology 198: 1691-1700.

AGNATI, L. F., M. ZOLI, I. STROMBERG and K. FUXE, 1995 INTERCELLULAR COMMUNICATION IN THE BRAIN - WIRING VERSUS VOLUME TRANSMISSION. Neuroscience 69: 711-726.

AGOSTO, J., J. C. CHOI, K. M. PARISKY, G. STILWELL, M. ROSBASH *et al.*, 2008 Modulation of GABAA receptor desensitization uncouples sleep onset and maintenance in Drosophila. Nat Neurosci 11: 354-359.

ALEKSEYENKO, O. V., C. LEE and E. A. KRAVITZ, 2010 Targeted Manipulation of Serotonergic Neurotransmission Affects the Escalation of Aggression in Adult Male *Drosophila melanogaster*. PLoS ONE 5: e10806.

ALEX, K. D., and E. A. PEHEK, 2007 Pharmacologic mechanisms of serotonergic regulation of dopamine neurotransmission. Pharmacology & Therapeutics 113: 296-320.

ANDO, R., H. HAMA, M. YAMAMOTO-HINO, H. MIZUNO and A. MIYAWAKI, 2002 An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. Proceedings of the National Academy of Sciences of the United States of America 99: 12651-12656.

ANDRETIC, R., B. VAN SWINDEREN and R. J. GREENSPAN, 2005 Dopaminergic Modulation of Arousal in Drosophila. Current Biology 15: 1165-1175.

ANSORGE, M. S., E. MORELLI and J. A. GINGRICH, 2008 Inhibition of Serotonin But Not Norepinephrine Transport during Development Produces Delayed, Persistent Perturbations of Emotional Behaviors in Mice. J. Neurosci. 28: 199-207.

ANSORGE, M. S., M. ZHOU, A. LIRA, R. HEN and J. A. GINGRICH, 2004 Early-Life Blockade of the 5-HT Transporter Alters Emotional Behavior in Adult Mice. Science 306: 879-881.

ARVAN, P., and D. CASTLE, 1998 Sorting and storage during secretory granule biogenesis: looking backward and looking forward. Biochem J 332: 593-610.

ATLURI, P. P., and T. A. RYAN, 2006 The Kinetics of Synaptic Vesicle Reacidification at Hippocampal Nerve Terminals. J. Neurosci. 26: 2313-2320.

AXELROD, J., and J. M. SAAVEDRA, 1977 Octopamine. Nature 265: 501-504.

BACON, J. P., K. S. THOMPSON and M. STERN, 1995 Identified octopaminergic neurons provide an arousal mechanism in the locust brain. Journal of Neurophysiology 74: 2739-2743.

BANERJEE, S., J. LEE, K. VENKATESH, C.-F. WU and G. HASAN, 2004 Loss of Flight and Associated Neuronal Rhythmicity in Inositol 1,4,5-Trisphosphate Receptor Mutants of Drosophila. The Journal of Neuroscience 24: 7869-7878.

BARNES, J. J. M., A. J. DEAN, L. S. NANDAM, R. G. O'CONNELL and M. A. BELLGROVE, 2011 The Molecular Genetics of Executive Function: Role of Monoamine System Genes. Biological Psychiatry In Press, Corrected Proof.

BARRON, A. B., E. SOVIK and J. L. CORNISH, 2010 The roles of dopamine and related compounds in reward seeking behaviour across animal phyla. Frontiers in Behavioral Neuroscience 4.

BAUDOUX, S., C. DUCH and O. T. MORRIS, 1998 Coupling of Efferent Neuromodulatory Neurons to Rhythmical Leg Motor Activity in the Locust. Journal of Neurophysiology 79: 361-370.

BAYERSDORFER, F., A. VOIGT, S. SCHNEUWLY and J. A. BOTELLA, 2010 Dopamine-dependent neurodegeneration in Drosophila models of familial and sporadic Parkinson's disease. Neurobiology of Disease 40: 113-119.

BECNEL, J., O. JOHNSON, J. LUO, D. R. NÄSSEL and C. D. NICHOLS, 2011 The Serotonin 5-HT_7Dro Receptor Is Expressed in the Brain of Drosophila, and Is Essential for Normal Courtship and Mating. PLoS ONE 6: e20800.

BELLO, B., D. RESENDEZ-PEREZ and W. J. GEHRING, 1998 Spatial and temporal targeting of gene expression in Drosophila by means of a tetracycline-dependent transactivator system. Development 125: 2193-2202.

BICKER, G., 1999 Biogenic amines in the brain of the honeybee: Cellular distribution, development, and behavioral functions, pp. 166-178. John Wiley & Sons, Inc.

BLENAU, W., and A. BAUMANN, 2001 Molecular and pharmacological properties of insect biogenic amine receptors: Lessons from Drosophila melanogaster and Apis mellifera. Archives of Insect Biochemistry and Physiology 48: 13-38.

BONIFACINO, J. S., and L. M. TRAUB, 2003 SIGNALS FOR SORTING OF TRANSMEMBRANE PROTEINS TO ENDOSOMES AND LYSOSOMES *. Annual Review of Biochemistry 72: 395-447.

BOUREAU, Y. L., and P. DAYAN, 2010 Opponency Revisited: Competition and Cooperation Between Dopamine and Serotonin. Neuropsychopharmacology 36: 74-97.

BRAND, A. H., and N. PERRIMON, 1993 Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development 118: 401-415.

BRENNER, S., 1974 THE GENETICS OF CAENORHABDITIS ELEGANS. Genetics 77: 71-94.

BROMBERG-MARTIN, E. S., M. MATSUMOTO and O. HIKOSAKA, 2010 Dopamine in Motivational Control: Rewarding, Aversive, and Alerting. Neuron 68: 815-834.

BROTZ, T., and A. BORST, 1996 Cholinergic and GABAergic receptors on fly tangential cells and their role in visual motion detection. J Neurophysiol 76: 1786-1799.

BROTZ, T., E. D. GUNDELFINGER and A. BORST, 2001 Cholinergic and GABAergic pathways in fly motion vision. BMC Neurosci 2.

BRUNK, I., C. BLEX, S. RACHAKONDA, M. HOLTJE, S. WINTER *et al.*, 2006 The first luminal domain of vesicular monoamine transporters mediates G-protein-dependent regulation of transmitter uptake. Journal of Biological Chemistry 281: 33373-33385.

BUDNIK, V., L. MARTIN-MORRIS and K. WHITE, 1986 Perturbed pattern of catecholamine-containing neurons in mutant Drosophila deficient in the enzyme dopa decarboxylase. The Journal of Neuroscience 6: 3682-3691.

BUDNIK, V., C. F. WU and K. WHITE, 1989 Altered branching of serotonin-containing neurons in Drosophila mutants unable to synthesize serotonin and dopamine. J. Neurosci. 9: 2866-2877.

BÜLTHOFF, H., and I. BÜLTHOFF, 1987 GABA-antagonist inverts movement and object detection in flies. Brain Research 407: 152-158.

BUNIN, M. A., and R. M. WIGHTMAN, 1998 Quantitative evaluation of 5-hydroxytryptamine (serotonin) neuronal release and uptake: An investigation of extrasynaptic transmission. Journal of Neuroscience 18: 4854-4860.

CASEY, J. R., S. GRINSTEIN and J. ORLOWSKI, 2010 Sensors and regulators of intracellular pH. Nat Rev Mol Cell Biol 11: 50-61.

CASPI, A., J. MCCLAY, T. E. MOFFITT, J. MILL, J. MARTIN *et al.*, 2002 Role of Genotype in the Cycle of Violence in Maltreated Children. Science 297: 851-854.

CASPI, A., T. E. MOFFITT, M. CANNON, J. MCCLAY, R. MURRAY *et al.*, 2005 Moderation of the Effect of Adolescent-Onset Cannabis Use on Adult Psychosis by a Functional Polymorphism in the Catechol-O-Methyltransferase Gene: Longitudinal Evidence of a Gene X Environment Interaction. Biological Psychiatry 57: 1117-1127.

CASPI, A., K. SUGDEN, T. E. MOFFITT, A. TAYLOR, I. W. CRAIG *et al.*, 2003 Influence of Life Stress on Depression: Moderation by a Polymorphism in the 5-HTT Gene. Science 301: 386-389.

CERTEL, S. J., M. G. SAVELLA, D. C. F. SCHLEGEL and E. A. KRAVITZ, 2007 Modulation of Drosophila male behavioral choice. Proceedings of the National Academy of Sciences 104: 4706-4711.

CHADA, S. R., and P. J. HOLLENBECK, 2004 Nerve Growth Factor Signaling Regulates Motility and Docking of Axonal Mitochondria. Current Biology 14: 1272-1276.

CHANG, H.-Y., A. GRYGORUK, E. S. BROOKS, L. C. ACKERSON, N. T. MAIDMENT *et al.*, 2006 Over-expression of the Drosophila vesicular monoamine transporter increases motor activity and courtship but decreases the behavioral response to cocaine. Molecular Psychiatry 11: 99-113.

CHASE, D., and M. KOELLE, 2007 Biogenic amine neurotransmitters in C. elegans. WormBook Feb 20: 1-15.

CHEN, M. S., R. A. OBAR, C. C. SCHROEDER, T. W. AUSTIN, C. A. POODRY *et al.*, 1991 Multiple forms of dynamin are encoded by shibire, a Drosophila gene involved in endocytosis. Nature 351: 583-586.

CHUDAKOV, D. M., V. V. VERKHUSHA, D. B. STAROVEROV, E. A. SOUSLOVA, S. LUKYANOV *et al.*, 2004 Photoswitchable cyan fluorescent protein for protein tracking. Nature Biotechnology 22: 1435-1439.

CLAGUE, M. J., S. URBE, F. ANIENTO and J. GRUENBERG, 1994 Vacuolar ATPase activity is required for endosomal carrier vesicle formation. Journal of Biological Chemistry 269: 21-24.

COLAS, J.-F., J.-M. LAUNAY, J.-L. VONESCH, P. HICKEL and L. MAROTEAUX, 1999 Serotonin synchronises convergent extension of ectoderm with morphogenetic gastrulation movements in Drosophila. Mechanisms of Development 87: 77-91.

COLE, S. H., G. E. CARNEY, C. A. MCCLUNG, S. S. WILLARD, B. J. TAYLOR *et al.*, 2005 Two Functional but Noncomplementing Drosophila Tyrosine Decarboxylase Genes: DISTINCT ROLES FOR NEURAL TYRAMINE AND OCTOPAMINE IN FEMALE FERTILITY. J. Biol. Chem. 280: 14948-14955.

COLLIVER, T. L., S. J. PYOTT, M. ACHALABUN and A. G. EWING, 2000 VMAT-Mediated Changes in Quantal Size and Vesicular Volume. J. Neurosci. 20: 5276-5282.

COOMBE, P., and M. HEISENBERG, 1986 The structural brain mutant Vacuolar medulla of Drosophila melanogaster with specific behavioral defects and cell degeneration in the adult. J Neurogenetics 3: 135-158.

COON, S. L., and D. B. BONAR, 1986 Norepinephrine and Dopamine Content of Larvae and Spat of the Pacific Oyster, *Crassostrea Gigas*. Biol Bull 171: 632-639.

COOPER, R. L., and W. S. NECKAMEYER, 1999 Dopaminergic modulation of motor neuron activity and neuromuscular function in Drosophila melanogaster. Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology 122: 199-210.

CREMONA, O., G. DI PAOLO, M. R. WENK, A. LÃ¼THI, W. T. KIM *et al.*, 1999 Essential Role of Phosphoinositide Metabolism in Synaptic Vesicle Recycling. Cell 99: 179-188.

CROCKER, A., and A. SEHGAL, 2008 Octopamine Regulates Sleep in Drosophila through Protein Kinase A-Dependent Mechanisms. J. Neurosci. 28: 9377-9385.

CROCKER, A., M. SHAHIDULLAH, I. B. LEVITAN and A. SEHGAL, 2010 Identification of a Neural Circuit that Underlies the Effects of Octopamine on Sleep:Wake Behavior. Neuron 65: 670-681.

CROFT, B. G., G. D. FORTIN, A. T. CORERA, R. H. EDWARDS, A. BEAUDET *et al.*, 2005 Normal Biogenesis and Cycling of Empty Synaptic Vesicles in Dopamine Neurons of Vesicular Monoamine Transporter 2 Knockout Mice. Mol Biol Cell 16: 306-315.

DANIELS, R. W., C. A. COLLINS, K. CHEN, M. V. GELFAND, D. E. FEATHERSTONE *et al.*, 2006 A single vesicular glutamate transporter is sufficient to fill a synaptic vesicle. Neuron 49: 11-16.

DANIELS, R. W., C. A. COLLINS, M. V. GELFAND, J. DANT, E. S. BROOKS *et al.*, 2004 Increased Expression of the Drosophila Vesicular Glutamate Transporter Leads to Excess Glutamate Release and a Compensatory Decrease in Quantal Content. J. Neurosci. 24: 10466-10474.

DASARI, S., and R. L. COOPER, 2004 Modulation of sensory-CNS-motor circuits by serotonin, octopamine, and dopamine in semi-intact Drosophila larva. Neuroscience Research 48: 221-227.

DAUBERT, E. A., and B. G. CONDRON, 2010 Serotonin: a regulator of neuronal morphology and circuitry. Trends in Neurosciences 33: 424-434.

DAUBERT, E. A., D. S. HEFFRON, J. W. MANDELL and B. G. CONDRON, 2010 Serotonergic dystrophy induced by excess serotonin. Molecular and Cellular Neuroscience 44: 297-306.

DE-MIGUEL, F. F., and C. TRUETA, 2005 Synaptic and extrasynaptic secretion of serotonin. Cellular and Molecular Neurobiology 25: 297-312.

DE CAMILLI, P., and R. JAHN, 1990 Pathways to Regulated Exocytosis in Neurons. Annual Review of Physiology 52: 625-645.

DE WIT, J., R. F. TOONEN and M. VERHAGE, 2009 Matrix-Dependent Local Retention of Secretory Vesicle Cargo in Cortical Neurons. Journal of Neuroscience 29: 23-37.

DEL CASTILLO, J., and B. KATZ, 1954 Quantal components of the end-plate potential. J Physiol 124: 560-573.

DI MATTEO, V., M. CACCHIO, C. DI GIULIO and E. ESPOSITIO, 2001 Role of serotonin$_{2C}$ receptors in the control of brain dopaminergic function. Pharmacology Biochemistry and Behavior 71: 727-734.

DICKMAN, D. K., J. A. HORNE, I. A. MEINERTZHAGEN and T. L. SCHWARZ, 2005 A Slowed Classical Pathway Rather Than Kiss-and-Run Mediates Endocytosis at Synapses Lacking Synaptojanin and Endophilin. Cell 123: 521-533.

DOUGLASS, J., and N. STAUSFELD, 1995 Visual motion detection circuits in flies: peripheral motion computation by identified small-field retinotopic neurons. J Neurosci 15: 5596-5611.

DRAPER, I., P. T. KURSHAN, E. MCBRIDE, F. R. JACKSON and A. S. KOPIN, 2007 Locomotor activity is regulated by D2-like receptors in Drosophila: An anatomic and functional analysis. Developmental Neurobiology 67: 378-393.

DUCLOS, S., R. CORSINI and M. DESJARDINS, 2003 Remodeling of endosomes during lysosome biogenesis involves `kiss and run' fusion events regulated by rab5. Journal of Cell Science 116: 907-918.

DUERR, J. S., D. L. FRISBY, J. GASKIN, A. DUKE, K. ASERMELY *et al.*, 1999 The cat-1 Gene of Caenorhabditis elegans Encodes a Vesicular Monoamine Transporter Required for Specific Monoamine-Dependent Behaviors. J. Neurosci. 19: 72-84.

EDWARDS, R. H., 2007 The Neurotransmitter Cycle and Quantal Size. Neuron 55: 835-858.

EGELHAAF, M., and A. BORST, 1993 A look into the cockpit of the fly: visual orientation, algorithms, and identified neurons. J Neurosci 13: 4563-4574.

ELEY, T. C., K. SUGDEN, A. CORSICO, A. M. GREGORY, P. SHAM *et al.*, 2004 Gene-environment interaction analysis of serotonin system markers with adolescent depression. Mol Psychiatry 9: 908-915.

EMELYANOV, A., and S. PARINOV, 2008 Mifepristone-inducible LexPR system to drive and control gene expression in transgenic zebrafish. Developmental Biology 320: 113-121.

ENG OOI, C., J. E. MOREIRA, E. C. DELL'ANGELICA, G. POY, D. A. WASSARMAN *et al.*, 1997 Altered expression of a novel adaptin leads to defective pigment granule biogenesis in the Drosophila eye color mutant garnet. EMBO J 16: 4508-4518.

ERICKSON, J. D., M. K. H. SCHAFER, T. I. BONNER, L. E. EIDEN and E. WEIHE, 1996 Distinct pharmacological properties and distribution in neurons and endocrine cells of two isoforms of the human vesicular monoamine transporter. Proceedings of the National Academy of Sciences of the United States of America 93: 5166-5171.

EVANS, P. R., and D. J. OWEN, 2002 Endocytosis and vesicle trafficking. Current Opinion in Structural Biology 12: 814-821.

FAROOQUI, T., 2007 Octopamine-Mediated Neuromodulation of Insect Senses. Neurochemical Research 32: 1511-1529.

FATT, P., and B. KATZ, 1952 Spontaneous subthreshold activity at motor nerve endings. J Physiol 117: 109-128.

FEATHERSTONE, D. E., E. M. RUSHTON, M. HILDERBRAND-CHAE, A. M. PHILLIPS, F. R. JACKSON *et al.*, 2000 Presynaptic Glutamic Acid Decarboxylase Is Required for Induction of the Postsynaptic Receptor Field at a Glutamatergic Synapse. Neuron 27: 71-84.

FEI, H., A. GRYGORUK, E. S. BROOKS, A. CHEN and D. E. KRANTZ, 2008 Trafficking of Vesicular Neurotransmitter Transporters. Traffic 9: 1425-1436.

FENG, Y., A. UEDA and C.-F. WU, 2004 A MODIFIED MINIMAL HEMOLYMPH-LIKE SOLUTION, HL3.1, FOR PHYSIOLOGICAL RECORDINGS AT THE NEUROMUSCULAR JUNCTIONS OF NORMAL AND MUTANT DROSOPHILA LARVAE. Journal of Neurogenetics 18: 377-402.

FLORESCO, S. B., A. R. WEST, B. ASH, H. MOORE and A. A. GRACE, 2003 Afferent modulation of dopamine neuron firing differentially regulates tonic and phasic dopamine transmission. Nat Neurosci 6: 968-973.

FON, E. A., E. N. POTHOS, B.-C. SUN, N. KILLEEN, D. SULZER et al., 1997 Vesicular Transport Regulates Monoamine Storage and Release but Is Not Essential for Amphetamine Action. Neuron 19: 1271-1283.

FORD, D., N. HOE, G. N. LANDIS, K. TOZER, A. LUU et al., 2007 Alteration of Drosophila life span using conditional, tissue-specific expression of transgenes triggered by doxycyline or RU486/Mifepristone. Experimental Gerontology 42: 483-497.

FOX, L. E., D. R. SOLL and C.-F. WU, 2006 Coordination and Modulation of Locomotion Pattern Generators in Drosophila Larvae: Effects of Altered Biogenic Amine Levels by the Tyramine beta Hydroxlyase Mutation. J. Neurosci. 26: 1486-1498.

FREMEAU, R. T., JR., K. KAM, T. QURESHI, J. JOHNSON, D. R. COPENHAGEN et al., 2004 Vesicular Glutamate Transporters 1 and 2 Target to Functionally Distinct Synaptic Release Sites. Science 304: 1815-1819.

FRERKING, M., and M. WILSON, 1996 Saturation of postsynaptic receptors at central synapses? Current Opinion in Neurobiology 6: 395-403.

FRIGGI-GRELIN, F., H. COULOM, M. MELLER, D. GOMEZ, J. HIRSH et al., 2003 Targeted gene expression in *Drosophila* dopaminergic cells using regulatory sequences from tyrosine hydroxylase. Journal of Neurobiology 54: 618-627.

FUXE, K., A. DAHLSTROM, M. HOISTAD, D. MARCELLINO, A. JANSSON et al., 2007 From the Golgi-Cajal mapping to the transmitter-based characterization of the neuronal networks leading to two modes of brain communication: Wiring and volume transmission. Brain Research Reviews 55: 17-54.

FUXE, K., A. DAHLSTROM, G. JONSSON, D. MARCELLINO, M. GUESCINI et al., 2009 The discovery of Central Monoamine Neurons Gave Volume Transmission to the Wired Brain. Prog Neurobiol.

FUXE, K., A. B. DAHLSTROM, G. JONSSON, D. MARCELLINO, M. GUESCINI et al., 2010 The discovery of central monoamine neurons gave volume transmission to the wired brain. Progress in Neurobiology 90: 82-100.

GAINETDINOV, R. R., and M. G. CARON, 2003 Monoamine transporters: From genes to behavior. Annual Review of Pharmacology and Toxicology 43: 261-284.

GAINETDINOV, R. R., W. C. WETSEL, S. R. JONES, E. D. LEVIN, M. JABER *et al.*, 1999 Role of serotonin in the paradoxical calming effect of psychostimulants on hyperactivity. Science 283: 397-401.

GASNIER, B., 2004 The SLC32 transporter, a key protein for the synaptic release of inhibitory amino acids. Pflügers Archiv European Journal of Physiology 447: 756-759.

GERVAIS, J., J.-J. SOGHOMONIAN, D. RICHARD and C. ROUILLARD, 1999 Dopamine and Serotonin Interactions in the Modulation of the Expression of the Immediate-early Transcription Factor, Nerve Growth Factor-inducible B, in the Striatum. Neuroscience 91: 1045-1054.

GORDON, S., and M. H. DICKINSON, 2006 Role of calcium in the regulation of mechanical power in insect flight. Proceedings of the National Academy of Sciences of the United States of America 103: 4311-4315.

GRAVOTTA, L., A. GAVRILA, S. HOOD and S. AMIR, 2011 Global Depletion of Dopamine Using Intracerebroventricular 6-Hydroxydopamine Injection Disrupts Normal Circadian Wheel-Running Patterns and PERIOD2 Expression in the Rat Forebrain. Journal of Molecular Neuroscience: 1-10.

GREENSPAN, R. J., 2000 Courtship in *Drosophila*. Annual Review of Genetics 34: 205-232.

GREER, C. L., A. GRYGORUK, D. E. PATTON, B. LEY, R. ROMERO-CALDERON *et al.*, 2005 A splice variant of the *Drosophila* vesicular monoamine transporter contains a conserved trafficking domain and functions in the storage of dopamine, serotonin, and octopamine. J Neurobio 64: 239-258.

GRILLNER, S., 2003 The motor infrastructure: from ion channels to neuronal networks. Nat Rev Neurosci 4: 573-586.

GRUNTENKO, N., E. KARPOVA, A. ALEKSEEV, N. CHENTSOVA, E. BOGOMOLOVA *et al.*, 2007 Effects of octopamine on reproduction, juvenile hormone metabolism, dopamine, and 20-hydroxyecdysone contents in Drosophila. Archives of Insect Biochemistry and Physiology 65: 85-94.

GRYGORUK, A., H. FEI, R. W. DANIELS, B. C. MILLER, A. CHEN *et al.*, 2010a Vesicular neurotransmitter transporter trafficking in vivo: Moving from cells to flies. Fly 4: 302-305.

GRYGORUK, A., H. FEI, R. W. DANIELS, B. R. MILLER, A. DIANTONIO *et al.*, 2010b A tyrosine-based motif localizes a Drosophila vesicular transporter to synaptic vesicles in vivo. Journal of Biological Chemistry 285: 6867-6878.

GUIARD, B. P., M. EL MANSARI and P. BLIER, 2008 Cross-Talk between Dopaminergic and Noradrenergic Systems in the Rat Ventral Tegmental Area, Locus Ceruleus, and Dorsal Hippocampus. Molecular Pharmacology 74: 1463-1475.

GURSKAYA, N. G., V. V. VERKHUSHA, A. S. SHCHEGLOV, D. B. STAROVEROV, T. V. CHEPURNYKH *et al.*, 2006 Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. Nature Biotechnology 24: 461-465.

HAENISCH, B., and H. BÖNISCH, 2011 Depression and antidepressants: Insights from knockout of dopamine, serotonin or noradrenaline re-uptake transporters. Pharmacology & Therapeutics 129: 352-368.

HALL, J. C., 1994 The mating of a fly. Science 264: 1702-1714.

HAMASAKA, Y., and D. R. NÄSSEL, 2006 Mapping of serotonin, dopamine, and histamine in relation to different clock neurons in the brain of *Drosophila*. The Journal of Comparative Neurology 494: 314-330.

HANNAH, M. J., A. A. SCHMIDT and W. B. HUTTNER, 1999 SYNAPTIC VESICLE BIOGENESIS. Annual Review of Cell and Developmental Biology 15: 733-798.

HARDIE, S. L., and J. HIRSH, 2006 An improved method for the separation and detection of biogenic amines in adult Drosophila brain extracts by high performance liquid chromatography. Journal of Neuroscience Methods 153: 243-249.

HARDIE, S. L., J. X. ZHANG and J. HIRSH, 2007 Trace amines differentially regulate adult locomotor activity, cocaine sensitivity, and female fertility in *Drosophila melanogaster*. Developmental Neurobiology 67: 1396-1405.

HARRIS, T. W., E. HARTWIEG, H. R. HORVITZ and E. M. JORGENSEN, 2000 Mutations in Synaptojanin Disrupt Synaptic Vesicle Recycling. J Cell Biol 150: 589-600.

HARRISON, J. B., H. H. CHEN, E. SATTELLE, P. J. BARKER, N. S. HUSKISSON *et al.*, 1996 Immunocytochemical mapping of a C-terminus anti-peptide antibody to the GABA receptor subunit, RDL in the nervous system of Drosophila melanogaster. Cell Tissue Res 284: 269-278.

HARTMANN, M., R. HEUMANN and V. LESSMANN, 2001 Synaptic secretion of BDNF after high-frequency stimulation of glutamatergic synapses. EMBO J 20: 5887-5897.

HASHEMZADEH-GARGARI, H., and W. OTTO FRIESEN, 1989 Modulation of swimming activity in the medicinal leech by serotonin and octopamine. Comparative Biochemistry and Physiology Part C: Comparative Pharmacology 94: 295-302.

HIRASHIMA, A., M. J. SUKHANOVA and I. Y. RAUSCHENBACH, 2000 Genetic Control of Biogenic-Amine Systems in *Drosophila* Under Normal and Stress Conditions. Biochemical Genetics 38: 163-176.

HIRSH, J., T. RIEMENSPERGER, H. COULOM, M. ICHÉ, J. COUPAR *et al.*, 2010 Roles of Dopamine in Circadian Rhythmicity and Extreme Light Sensitivity of Circadian Entrainment. Current Biology 20: 209-214.

HODGETTS, R. B., and S. L. O'KEEFE, 2006 DOPA DECARBOXYLASE: A Model Gene-Enzyme System for Studying Development, Behavior, and Systematics. Annual Review of Entomology 51: 259-284.

HOMBERG, U., T. KINGAN and J. HILDEBRAND, 1987 Immunocytochemistry of GABA in the brain and suboesophageal ganglion of Manduca sexta. Cell Tissue Res 248: 1-24.

HORTON, A. C., and M. D. EHLERS, 2003 Neuronal Polarity and Trafficking. Neuron 40: 277-295.

HOSÁK, L., 2007 Role of the COMT gene Val158Met polymorphism in mental disorders: A review. European Psychiatry 22: 276-281.

HSOUNA, A., H. O. LAWAL, I. IZEVBAYE, T. HSU and J. M. O'DONNELL, 2007 Drosophila dopamine synthesis pathway genes regulate tracheal morphogenesis. Dev Biol 308: 30-43.

HUA, Y., R. SINHA, C. S. THIEL, R. SCHMIDT, J. HUVE et al., 2011 A readily retrievable pool of synaptic vesicles. Nat Neurosci 14: 833-839.

HURLBUT, W. P., N. IEZZI, R. FESCE and B. CECCARELLI, 1990 Correlation between quantal secretion and vesicle loss at the frog neuromuscular junction. J Physiol 425: 501-526.

ISHIKAWA, T., Y. SAHARA and T. TAKAHASHI, 2002 A Single Packet of Transmitter Does Not Saturate Postsynaptic Glutamate Receptors. Neuron 34: 613-621.

JAFFE, E. H., A. MARTY, A. SCHULTE and R. H. CHOW, 1998 Extrasynaptic Vesicular Transmitter Release from the Somata of Substantia Nigra Neurons in Rat Midbrain Slices. The Journal of Neuroscience 18: 3548-3553.

JOSHUA, M., A. ADLER and H. BERGMAN, 2009 The dynamics of dopamine in control of motor behavior. Current Opinion in Neurobiology 19: 615-620.

KANNER, B. I., 2006 Structure and funciton of sodium-copuled GABA and glutamate transporters. J Membr Biol 213: 89-100.

KELLY, 1993 Storage and release of neurotransmitters. Cell 72/10: 43-53.

KIDOKORO, Y., 2006 Vesicle Trafficking and Recycling at The Neuromuscular Junction: Two Pathways for Endocytosis, pp. 145-164 in International Review of Neurobiology. Academic Press.

KIDOKORO, Y., H. KUROMI, R. DELGADO, C. MAUREIRA, C. OLIVA et al., 2004 Synaptic vesicle pools and plasticity of synaptic transmission at the Drosophila synapse. Brain Res Rev 47: 18-32.

KIM, S. M., V. KUMAR, Y.-Q. LIN, S. KARUNANITHI and M. RAMASWAMI, 2009 Fos and Jun potentiate individual release sites and mobilize the reserve synaptic-vesicle pool at the Drosophila larval motor synapse. Proceedings of the National Academy of Sciences 106: 4000-4005.

KITAMOTO, T., 2002 TARGETED EXPRESSION OF TEMPERATURE-SENSITIVE DYNAMIN TO STUDY NEURAL MECHANISMS OF COMPLEX BEHAVIOR IN Drosophila. Journal of Neurogenetics 16: 205 - 228.

KOBAYASHI, K., S. MORITA, H. SAWADA, T. MIZUGUCHI, K. YAMADA *et al.*, 1995 TARGETED DISRUPTION OF THE TYROSINE-HYDROXYLASE LOCUS RESULTS IN SEVERE CATECHOLAMINE DEPLETION AND PERINATAL LETHALITY IN MICE. Journal of Biological Chemistry 270: 27235-27243.

KOENIG, J. H., and K. IKEDA, 1989 Disappearance and reformation of synaptic vesicle membrane upon transmitter release observed under reversible blockage of membrane retrieval. J. Neurosci. 9: 3844-3860.

KOH, T.-W., P. VERSTREKEN and H. J. BELLEN, 2004 Dap160/Intersectin Acts as a Stabilizing Scaffold Required for Synaptic Development and Vesicle Endocytosis. Neuron 43: 193-205.

KOON, A. C., J. ASHLEY, R. BARRIA, S. DASGUPTA, R. BRAIN *et al.*, 2011

Autoregulatory and paracrine control of synaptic and behavioral plasticity by octopaminergic signaling. Nat Neurosci 14: 190-199.

KRANTZ, D. E., C. WAITES, V. OORSCHOT, Y. LIU, R. I. WILSON *et al.*, 2000 A Phosphorylation Site Regulates Sorting of the Vesicular Acetylcholine Transporter to Dense Core Vesicles. J. Cell Biol. 149: 379-396.

KRETZSCHMAR, D., B. POECK, H. ROTH, R. ERNST, A. KELLER *et al.*, 2000 Defective Pigment Granule Biogenesis and Aberrant Behavior Caused by Mutations in the Drosophila AP-3ÃŸ Adaptin Gene ruby. Genetics 155: 213-223.

KULA-EVERSOLE, E., E. NAGOSHI, Y. SHANG, J. RODRIGUEZ, R. ALLADA *et al.*, 2010 Surprising gene expression patterns within and between PDF-containing circadian neurons in Drosophila. Proceedings of the National Academy of Sciences 107: 13497-13502.

KUME, K., S. KUME, S. K. PARK, J. HIRSH and F. R. JACKSON, 2005 Dopamine Is a Regulator of Arousal in the Fruit Fly. J. Neurosci. 25: 7377-7384.

KUROMI, H., and Y. KIDOKORO, 2000 Tetanic stimulation recruits vesicles from reserve pool via a cAMP-mediated process in Drosophila synapses. Neuron 27: 133-143.

LAI, S.-L., and T. LEE, 2006 Genetic mosaic with dual binary transcriptional systems in Drosophila. Nat Neurosci 9: 703-709.

LASBLEIZ, C., J.-F. FERVEUR and C. EVERAERTS, 2006 Courtship behaviour of Drosophila melanogaster revisited. Animal Behaviour 72: 1001-1012.

LEBESTKY, T., J.-S. C. CHANG, H. DANKERT, L. ZELNIK, Y.-C. KIM *et al.*, 2009 Two Different Forms of Arousal in Drosophila Are Oppositely Regulated by the Dopamine D1 Receptor Ortholog DopR via Distinct Neural Circuits. Neuron 64: 522-536.

LEE, H.-G., S. ROHILA and K.-A. HAN, 2009 The Octopamine Receptor OAMB Mediates Ovulation via Ca2+ / Calmodulin-Dependent Protein Kinase II in the *Drosophila* Oviduct Epithelium. PLoS ONE 4: e4716.

LEE, H.-G., C.-S. SEONG, Y.-C. KIM, R. L. DAVIS and K.-A. HAN, 2003 Octopamine receptor OAMB is required for ovulation in Drosophila melanogaster. Developmental Biology 264: 179-190.

LEE, J. S., W.-K. HO and S.-H. LEE, 2012 Actin-dependent rapid recruitment of reluctant synaptic vesicles into a fast-releasing vesicle pool. Proceedings of the National Academy of Sciences 109: E765â€"E774.

LEGGIO, G. M., A. CATHALA, M. NENY, F. ROUGE-PONT, F. DRAGO *et al.*, 2009 *In vivo* evidence that constitutive activity of serotonin$_{2C}$ receptors in the medial prefrontal cortex participates in the control of dopamine release in the rat nucleus acumbens: differential effects of inverse agonist versus antagonist. Journal of Neurochemistry 111: 614-623.

LEITCH, B., and G. LAURENT, 1996 GABAergic synapses in the antennal lobe and mushroom body of the locus olfactory system. J Comp Neurol 372: 487-514.

LEVINE, J. D., P. FUNES, H. B. DOWSE and J. C. HALL, 2002 Signal analysis of behavioral and molecular cycles. BMC Neurosci 3.

LI, H., S. CHANEY, M. FORTE and J. HIRSH, 2000 Ectopic G-protein expression in dopamine and serotonin neurons blocks cocaine sensitization in Drosophila melanogaster. Current Biology 10: 211-214.

LI, H., C. L. WAITES, R. G. STAAL, Y. DOBRYY, J. PARK *et al.*, 2005 Sorting of Vesicular Monoamine Transporter 2 to the Regulated Secretory Pathway Confers the Somatodendritic Exocytosis of Monoamines. Neuron 48: 619-633.

LIMA, S. Q., and G. MIESENBÖCK, 2005 Remote Control of Behavior through Genetically Targeted Photostimulation of Neurons. Cell 121: 141-152.

LIU, T., L. DARTEVELLE, C. YUAN, H. WEI, Y. WANG *et al.*, 2008 Increased Dopamine Level Enhances Male-Male Courtship in Drosophila. The Journal of Neuroscience 28: 5539-5546.

LIU, T., L. DARTEVELLE, C. YUAN, H. WEI, Y. WANG *et al.*, 2009 Reduction of Dopamine Level Enhances the Attractiveness of Male *Drosophila* to Other Males. PLoS ONE 4: e4574.

LIU, X., W. C. KRAUSE and R. L. DAVIS, 2007 GABAA Receptor RDL Inhibits Drosophila Olfactory Associative Learning. Neuron 56: 1090-1102.

LIU, Y., and R. H. EDWARDS, 1997 The role of vesicular transport proteins in synaptic transmission and neural degeneration. Annual Review of Neuroscience 20: 125-156.

LIU, Y., D. PETER, A. ROGHANI, S. SCHULDINER, G. G. PRIVE *et al.*, 1992 A cDNA that suppresses MPP+ toxicity encodes a vesicular amine transporter. Cell 70: 539-551.

LOPEZ, A., L. DUPOU, A. ALTIBELLI, J. TROTARD and J. F. TOCANNE, 1988 Fluorescence recovery after photobleaching (FRAP) experiments under conditions of uniform disk illumination. Critical comparison of analytical solutions, and a new mathematical method for calculation of diffusion coefficient D. Biophysical Journal 53: 963-970.

MABRY, P. D., and B. A. CAMPBELL, 1973 Serotonergic inhibition of catecholamine-induced behavioral arousal. Brain Research 49: 381-391.

MACKEY, S., and T. CAREW, 1983 Locomotion in Aplysia: triggering by serotonin and modulation by bag cell extract. The Journal of Neuroscience 3: 1469-1477.

MAILMAN, R. B., and V. MURTHY, 2010 Third generation antipsychotic drugs: partial agonism or receptor functional selectivity. Curr Pharm Des 16: 488-501.

MARDER, E., 2007 Searching for Insight: Using Invertebrate Nervous Systems to Illuminate Fundamental Principles in Neuroscience, pp. 1-18 in *Invertebrate Neurobiology* edited by G. NORTH and R. J. GREENSPAN. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

MARDER, E., and D. BUCHER, 2001 Central pattern generators and the control of rhythmic movements. Current Biology 11: R986-R996.

MARKS, M. S., H. OHNO, T. KIRCHHAUSEN and J. S. BONIFACINO, 1997 Protein sorting by tyrosine-based signals adapting to the Ys and wherefores. Trends Cell Biol 7: 124-128.

McGUIRE, S. E., P. T. LE, A. J. OSBORN, K. MATSUMOTO and R. L. DAVIS, 2003 Spatiotemporal Rescue of Memory Dysfunction in Drosophila. Science 302: 1765-1768.

McINTIRE, S. L., R. J. REIMER, K. SCHUSKE, R. H. EDWARDS and E. M. JORGENSEN, 1997 Identification and characterization of the vesicular GABA transporter. Nature 389: 870-876.

MEIJER, J. H., and G. A. GROOS, 1988 Responsiveness of suprachiasmatic and ventral lateral geniculate neurons to serotonin and imipramine: A microiontophoretic study in normal and imipramine-treated rats. Brain Research Bulletin 20: 89-96.

MELTZER, H. Y., M. HUANG, V. D. M. GIUSEPPE DI GIOVANN and E. ENNIO, 2008 In vivo actions of atypical antipsychotic drug on serotonergic and dopaminergic systems, pp. 177-197 in *Progress in Brain Research*. Elsevier.

METAXAKIS, A., S. OEHLER, A. KLINAKIS and C. SAVAKIS, 2005 Minos as a Genetic and Genomic Tool in Drosophila melanogaster. Genetics 171: 571-581.

MEYER, E. P., C. MATUTE, P. STREIT and D. R. NÄSSEL, 1986 Insect optic lobe neurons identifiable with monoclonal antibodies to GABA. Histochemistry 84: 207-216.

MIDDLETON, C. A., U. NONGTHOMBA, K. PARRY, S. T. SWEENEY, J. C. SPARROW *et al.*, 2006 Neuromuscular organization and aminergic modulaiton of contractions in the *Drosophila* ovary. BMC Biology 4.

MIESENBOCK, G., D. A. DE ANGELIS and J. E. ROTHMAN, 1998 Visualizing secretion and synaptic transmission with pH-sensitive green fluorescent proteins. Nature 394: 192-195.

MOFFITT, T. E., A. CASPI and M. RUTTER, 2005 Strategy for Investigating Interations Between Measured Genes and Measured Environments. Arch Gen Psychiatry 62: 473-481.

MONASTIRIOTI, M., 2003 Distinct octopamine cell population residing in the CNS abdominal ganglion controls ovulation in Drosophila melanogaster. Developmental Biology 264: 38-49.

MONASTIRIOTI, M., J. C. E. LINN and K. WHITE, 1996 Characterization of Drosophila Tyramine beta -Hydroxylase Gene and Isolation of Mutant Flies Lacking Octopamine. J. Neurosci. 16: 3900-3911.

MORIN, L. P., and J. BLANCHARD, 1991 Serotonergic modulation of the hamster wheelrunning rhythm: response to lighting conditions and food deprivation. Brain Research 566: 186-192.

MÖSSNER, R., R. SIMANTOV, A. MARX, K. P. LESCH and I. SEIF, 2006 Aberrant accumulation of serotonin in dopaminergic neurons. Neuroscience Letters 401: 49-54.

MULLER, C., S. VIRY, M. MIEHE, C. ANDRIAMAMPANDRY, D. AUNIS *et al.*, 2002 Evidence for a γ-hydroxybutyrate (GHB) uptake by rat brain synaptic vesicles. Journal of Neurochemistry 80: 899-904.

MURPHY, D. L., Q. LI, S. ENGEL, C. WICHEMS, A. ANDREWS *et al.*, 2001 Genetic perspectives on the serotonin transporter. Brain Research Bulletin 56: 487-494.

NAKATA, T., S. TERADA and N. HIROKAWA, 1998 Visualization of the dynamics of synaptic vesicles and plasma membrane proteins in living axons. J Cell Biol 140: 659-674.

NÄSSEL, D., and U. HOMBERG, 2006 Neuropeptides in interneurons of the insect brain. Cell and Tissue Research 326: 1-24.

NECKAMEYER, W. S., 1996 Multiple Roles for Dopamine in *Drosophila* Development. Developmental Biology 176: 209-219.

NECKAMEYER, W. S., 1998a Dopamine and Mushroom Bodies in Drosophila: Experience-Dependent and -Independent Aspects of Sexual Behavior. Learning & Memory 5: 157-165.

NECKAMEYER, W. S., 1998b Dopamine modulates female sexual receptivity in Drosophila melanogaster. J Neurogenetics 12: 101-114.

NECKAMEYER, W. S., 2010 A trophic role for serotonin in the development of a simple feeding circuit. Dev Neurosci 32: 217-237.

NECKAMEYER, W. S., and W. G. QUINN, 1989 Isolation and characterization of the gene for drosophila tyrosine hydroxylase. Neuron 2: 1167-1175.

NECKAMEYER, W. S., and J. S. WEINSTEIN, 2005 Stress affects dopaminergic signaling pathways in *Drosophila melanogaster*. Stress 8: 117 - 131.

NEUSER, K., T. TRIPHAN, M. MRONZ, B. POECK and R. STRAUSS, 2008 Analysis of a spatial orientation memory in Drosophila. Nature 453: 1244-1247.

NIRENBERG, M. J., J. CHAN, Y. J. LIU, R. H. EDWARDS and V. M. PICKEL, 1996 Ultrastructural localization of the vesicular monoamine transporter-2 in midbrain dopaminergic neurons: Potential sites for somatodendritic storage and release of dopamine. Journal of Neuroscience 16: 4135-4145.

NITABACH, M. N., and P. H. TAGHERT, 2008 Organization of the Drosophila Circadian Control Circuit. Current Biology 18: R84-R93.

NUSBAUM, M. P., 1986 Synaptic basis of swim initiation in the leech. III. Synaptic effects of serotonin-containing interneurones (cells 21 and 61) on swim CPG neurones (cells 18 and 208). Journal of Experimental Biology 122: 303-321.

OH, S.-W., T. KINGSLEY, H.-H. SHIN, Z. ZHENG, H.-W. CHEN *et al.*, 2003 A P-element insertion screen identified mutations in 455 novel essential genes in *Drosophila*. Genetics 163: 195-201.

OHNO, H., J. STEWART, M. C. FOURNIER, H. BOSSHART, I. RHEE *et al.*, 1995 Interaciton of tyrosine-based sorting signals with clathrin-associated proteins. Science 269: 1872-1875.

OLSEN, S. R., and R. I. WILSON, 2008 Lateral presynaptic inhibition mediates gain control in an olfactory circuit. Nature 452: 956-960.

ORCHARD, I., J.-M. RAMIREZ and A. B. LANGE, 1993 A Multifunctional Role for Octopamine in Locust Flight. Annu Rev Entomol 38: 227-249.

OVERLY, C. C., H. I. RIEFF and P. J. HOLLENBECK, 1996 Organelle motility and metabolism in axons vs dendrites of cultured hippocampal neurons. Journal of Cell Science 109: 971-980.

PALADE, G. E., and S. L. PALAY, 1954 Electron microscope observations of interneuronal and neuromuscular synapses. Anat Rec 118: 335-336.

PARK, J., S. B. LEE, S. LEE, Y. KIM, S. SONG *et al.*, 2006 Mitochondrial dysfunction in Drosophila PINK1 mutants is complemented by parkin. Nature 441: 1157-1161.

PAROUTIS, P., N. TOURET and S. GRINSTEIN, 2004 The pH of the secretory pathway: Measurement, determinants, and regulation. Physiology 19: 207-215.

PENDLETON, R., A. RASHEED, T. SARDINA, T. TULLY and R. HILLMAN, 2002 Effects of tyrosine hydroxylase mutants on locomotor activity in Drosophila: a study in functional genomics. Behav Genet 32: 89-94.

PENDLETON, R. G., N. ROBINSON, R. ROYCHOWDHURY, A. RASHEED and R. HILLMAN, 1996 Reproduction and development in are dependent upon catecholamines. Life Sciences 59: 2083-2091.

PETER, D., Y. LIU, C. STERNINI, R. DE GIORGIO, N. BRECHA *et al.*, 1995 Differential expression of two vesicular monoamine transporters. J. Neurosci. 15: 6179-6188.

PHAIR, R. D., and T. MISTELI, 2001 Kinetic modelling approaches to in vivo imaging. Nat Rev Mol Cell Biol 2: 898-907.

POSKANZER, K. E., and G. W. DAVIS, 2004 Mobilization and fusion of a non-recycling pool of synaptic vesicles under conditions of endocytic blockade. Neuropharmacology 47: 714-723.

POSKANZER, K. E., K. W. MAREK, S. T. SWEENEY and G. W. DAVIS, 2003 Synaptotagmin I is necessary for compensatory synaptic vesicle endocytosis in vivo. Nature 426: 559-563.

POTHOS, E. N., K. E. LARSEN, D. E. KRANTZ, Y.-J. LIU, J. W. HAYCOCK *et al.*, 2000 Synaptic Vesicle Transporter Expression Regulates Vesicle Phenotype and Quantal Size. J. Neurosci. 20: 7297-7306.

PRADO, V., and M. PRADO, 2002 Signals invovled in targeting membrane proteins to synaptic vesicles. Cell Mol Neurobiol 22: 565-577.

RAHAMIMOFF, R., and J. M. FERNANDEZ, 1997 Pre- and Postfusion Regulation of Transmitter Release. Neuron 18: 17-27.

RAMIREZ, J. M., and K. G. PEARSON, 1991 OCTOPAMINERGIC MODULATION OF INTERNEURONS IN THE FLIGHT SYSTEM OF THE LOCUST. Journal of Neurophysiology 66: 1522-1537.

REGNIER-VIGOUROUX, A., S. A. TOOZE and W. B. HUTTNER, 1991 Newly synthesized synaptophysin is transported to synaptic-like microvesicles via constitutive secretory vesicles and the plasma membrane. EMBO J 10: 3589-3601.

REIFF, D. F., A. IHRING, G. GUERRERO, E. Y. ISACOFF, M. JOESCH *et al.*, 2005 In Vivo Performance of Genetically Encoded Indicators of Neural Activity in Flies. J. Neurosci. 25: 4766-4778.

REITS, E. A. J., and J. J. NEEFJES, 2001 From fixed to FRAP: measuring protein mobility and activity in living cells. Nature Cell Biology 3: E145-E147.

REMINGTON, S. J., 2006 Fluorescent proteins: maturation, photochemistry and photophysics. Current Opinion in Structural Biology 16: 714-721.

RICE, M. E., and S. J. CRAGG, 2008 Dopamine spillover after quantal release: Rethinking dopamine transmission in the nigrostriatal pathway. Brain Research Reviews 58: 303-313.

RIEGER, D., R. STANEWSKY and C. HELFRICH-FÖRSTER, 2003 Cryptochrome, Compound Eyes, Hofbauer-Buchner Eyelets, and Ocelli Play Different Roles in the Entrainment and Masking Pathway of the Locomotor Activity Rhythm in the Fruit Fly Drosophila Melanogaster. Journal of Biological Rhythms 18: 377-391.

RIEMENSPERGER, T., G. ISABEL, H. L. N. COULOM, K. NEUSER, L. SEUGNET et al., 2011 Behavioral consequences of dopamine deficiency in the Drosophila central nervous system. Proceedings of the National Academy of Sciences 108: 834-839.

RIND, F. C., R. D. SANTER and G. A. WRIGHT, 2008 Arousal Facilitates Collision Avoidance Mediated by a Looming Sensitive Visual Neuron in a Flying Locust. Journal of Neurophysiology 100: 670-680.

RINGSTAD, N., H. GAD, P. LOW, G. DI PAOLO, L. BRODIN et al., 1999 Endophilin/SH3p4 Is Required for the Transition from Early to Late Stages in Clathrin-Mediated Synaptic Vesicle Endocytosis. Neuron 24: 143-154.

RISTER, J., D. PAULS, B. SCHNELL, C.-Y. TING, C.-H. LEE et al., 2007 Dissection of the Peripheral Motion Channel in the Visual System of Drosophila melanogaster. Neuron 56: 155-170.

RIZZOLI, S. O., I. BETHANI, D. ZWILLING, D. WENZEL, T. J. SIDDIQUI et al., 2006 Evidence for Early Endosome-like Fusion of Recently Endocytosed Synaptic Vesicles. Traffic 7: 1163-1176.

RIZZOLI, S. O., and W. J. BETZ, 2005 SYNAPTIC VESICLE POOLS. Nature Reviews Neuroscience 6: 57-69.

ROBINSON, M. S., 2004 Adaptable adaptors for coated vesicles. Trends in Cell Biology 14: 167-174.

RODRÍGUEZ-VALENTÍN, R., I. LÓPEZ-GONZÁLEZ, R. JORQUERA, P. LABARCA, M. ZURITA et al., 2006 Oviduct contraction in Drosophila is modulated by a neural network that is both, octopaminergic and glutamatergic. Journal of Cellular Physiology 209: 183-198.

ROEDER, T., 1999 Octopamine in invertebrates. Progress in Neurobiology 59: 533-561.

ROEDER, T., 2005 TYRAMINE AND OCTOPAMINE: Ruling Behavior and Metabolism. Annual Review of Entomology 50: 447-477.

ROMERO-CALDERON, R., G. UHLENBROCK, J. BORYCZ, A. F. SIMON, A. GRYGORUK et al., 2008 A Glial Variant of the Vesicular Monoamine Transporter Is Required To Store Histamine in the Drosophila Visual System. PLoS Genet 4: e1000245.

SAGNÉ, C., S. EL MESTIKAWY, M.-F. ISAMBERT, M. HAMON, J.-P. HENRY et al., 1997 Cloning of a functional vesicular GABA and glycine transporter by screening of genome databases. FEBS Letters 417: 177-183.

SAKABA, T., and E. NEHER, 2001 Calmodulin Mediates Rapid Recruitment of Fast-Releasing Synaptic Vesicles at a Calyx-Type Synapse. Neuron 32: 1119-1131.

SANKARANARAYANAN, S., D. DE ANGELIS, J. E. ROTHMAN and T. A. RYAN, 2000 The Use of pHluorins for Optical Measurements of Presynaptic Activity. Biophysical Journal 79: 2199-2208.

SANKARANARAYANAN, S., and T. A. RYAN, 2001 Calcium accelerates endocytosis of vSNAREs at hippocampal synapses. Nat Neurosci 4: 129-136.

SANTOS, M. S., J. BARBOSA, G. S. VELOSO, F. RIBEIRO, C. KUSHMERICK *et al.*, 2001 Trafficking of green fluorescent protein tagged-vesicular acetylcholine transporter to varicosities in a cholinergic cell line. Journal of Neurochemistry 78: 1104-1113.

SANYAL, S., R. F. WINTLE, K. S. KINDT, W. M. NUTTLEY, R. ARVAN *et al.*, 2004 Dopamine modulates the plasticity of mechanosensory responses in Caenorhabditis elegans. EMBO J 23: 473-482.

SARA, S. J., 2009 The locus coeruleus and noradrenergic modulation of cognition. Nat Rev Neurosci 10: 211-223.

SARASWATI, S., L. E. FOX, D. R. SOLL and C.-F. WU, 2004 Tyramine and octopamine have opposite effects on the locomotion of *Drosophila* larvae. Journal of Neurobiology 58: 425-441.

SARTER, M., J. P. BRUNO and V. PARIKH, 2007 Abnormal Neurotransmitter Release Underlying Behavioral and Cognitive Disorders: Toward Concepts of Dynamic and Function-Specific Dysregulation. Neuropsychopharmacology 32: 1452-1461.

SARTER, M., V. PARIKH and W. M. HOWE, 2009 Phasic acetylcholine release and the volume transmission hypothesis: time to move on. Nature Reviews Neuroscience 10: 383-390.

SAWIN, E. R., R. RANGANATHAN and H. R. HORVITZ, 2000 C. elegans Locomotory Rate Is Modulated by the Environment through a Dopaminergic Pathway and by Experience through a Serotonergic Pathway. Neuron 26: 619-631.

SCHAERLINGER, B., J. M. LAUNAY, J. L. VONESCH and L. MAROTEAUX, 2007 Gain of affinity point mutation in the serotonin receptor gene 5-HT2Dro accelerates germband extension movements during *Drosophila* gastrulation. Developmental Dynamics 236: 991-999.

SCHEINER, R., A. BAUMANN and W. BLENAU, 2006 Aminergic control and modulation of honeybee behavior. Curr Neuropharmacol 4: 259-276.

SCHOUSBOE, A., 2000 Pharmacological and Functional Characterization of Astrocytic GABA Transport: A Short Review. Neurochem. Res. 25: 1241-1233.

SCHULTZ, W., 2007 Multiple Dopamine Functions at Different Time Courses. Annual Review of Neuroscience 30: 259-288.

SCHULTZ, W., 2010 Dopamine signals for reward value and risk: basic and recent data. Behavioral and Brain Functions 6: 24.

SCHUSKE, K., A. A. BEG and E. M. JORGENSEN, 2004 The GABA nervous system in C. elegans. Trends in Neurosciences 27: 407-414.

SCHUSKE, K. R., J. E. RICHMOND, D. S. MATTHIES, W. S. DAVIS, S. RUNZ *et al.*, 2003 Endophilin Is Required for Synaptic Vesicle Endocytosis by Localizing Synaptojanin. Neuron 40: 749-762.

SCHWAERZEL, M., M. MONASTIRIOTI, H. SCHOLZ, F. FRIGGI-GRELIN, S. BIRMAN *et al.*, 2003 Dopamine and Octopamine Differentiate between Aversive and Appetitive Olfactory Memories in Drosophila. J. Neurosci. 23: 10495-10502.

SCHWEIZER, F. E., and T. A. RYAN, 2006 The synaptic vesicle: cycle of exocytosis and endocytosis. Current Opinion in Neurobiology 16: 298-304.

SEDORE WILLARD, S., C. M. KOSS and C. CRONMILLER, 2006 Chronic cocaine exposure in Drosophila: Life, cell death and oogenesis. Developmental Biology 296: 150-163.

SESACK, S. R., and A. A. GRACE, 2010 Cortico-Basal Ganglia Reward Network: Microcircuitry

Neuropsychopharmacology 35: 27-47.

SHAFER, O. T., and P. H. TAGHERT, 2009 RNA-Interference Knockdown of *Drosophila* Pigment Dispersing Factor in Neuronal Subsets: The Anatomical Basis of a Neuropeptide's Circadian Functions. PLoS ONE 4: e8298.

SHANER, N. C., G. H. PATTERSON and M. W. DAVIDSON, 2007 Advances in fluorescent protein technology. Journal of Cell Science 120: 4247-4260.

SHEEBA, V., K. J. FOGLE and T. C. HOLMES, 2010 Persistence of Morning Anticipation Behavior and High Amplitude Morning Startle Response Following Functional Loss of Small Ventral Lateral Neurons in Drosophila. PLoS ONE 5: e11628.

SHOHAMY, D., and R. A. ADCOCK, 2010 Dopamine and adaptive memory. Trends in Cognitive Sciences 14: 464-472.

SILBERING, A. F., and C. G. GALIZIA, 2007 Processing of Odor Mixtures in the *Drosophila* Antennal Lobe Reveals both Global Inhibition and Glomerulus-Specific Interactions. J Neurosci 27: 11966-11977.

SIMON, A. F., R. DANIELS, R. ROMERO-CALDERON, A. GRYGORUK, H.-Y. CHANG *et al.*, 2009 Drosophila Vesicular Monoamine Transporter Mutants Can Adapt to Reduced or Eliminated Vesicular Stores of Dopamine and Serotonin. Genetics 181: 525-541.

SINAKEVITCH, I., and N. J. STRAUSFELD, 2004 Chemical neuroanatomy of the fly's movement detection pathway. The Journal of Comparative Neurology 468: 6-23.

SITARAMAN, D., M. ZARS, H. LAFERRIERE, Y.-C. CHEN, A. SABLE-SMITH *et al.*, 2008 Serotonin is necessary for place memory in Drosophila. Proceedings of the National Academy of Sciences 105: 5579-5584.

SITARAMAN, D., M. ZARS and T. ZARS, 2010 Place memory formation in Drosophila is independent of proper octopamine signaling. Journal of Comparative Physiology A: Neuroethology, Sensory, Neural, and Behavioral Physiology 196: 299-305.

SOMBATI, S., and G. HOYLE, 1984 Generation of specific behaviors in a locust by local release into neuropil of the natural neuromodulator octopamine. Journal of Neurobiology 15: 481-506.

SORA, I., F. S. HALL, A. M. ANDREWS, M. ITOKAWA, X.-F. LI *et al.*, 2001 Molecular mechanisms of cocaine reward: Combined dopamine and serotonin transporter knockouts eliminate cocaine place preference. Proceedings of the National Academy of Sciences 98: 5300-5305.

SOUTHWICK, S. M., M. VYTHILINGAM and D. S. CHARNEY, 2005 The Psychobiology of Depression and Resilience to Stress: Implications for Prevention and Treatment*. Annual Review of Clinical Psychology 1: 255-291.

STEINERT, J. R., H. KUROMI, A. HELLWIG, M. KNIRR, A. W. WYATT *et al.*, 2006 Experience-Dependent Formation and Recruitment of Large Vesicles from Reserve Pool. Neuron 50: 723-733.

STERN, M., 1999 Octopamine in the locust brain: Cellular distribution and functional significance in an arousal mechanism, pp. 135-141. John Wiley & Sons, Inc.

SUH, J., and F. R. JACKSON, 2007 Drosophila Ebony Activity Is Required in Glia for the Circadian Regulation of Locomotor Activity. Neuron 55: 435-447.

SUN, B.-C., and P. SALVATERRA, 1995 Two Drosophila nervous system antigens, Nervana 1 and 2, are homologous to the beta subunit of $Na^+,K(+)$-ATPase. Proceedings of the National Academy of Sciences 92: 5396-5400.

SUO, S., J. G. CULOTTI and H. H. M. VAN TOL, 2009 Dopamine counteracts octopamine signalling in a neural circuit mediating food response in C. elegans. EMBO J 28: 2437-2448.

SUSTER, M. L., and M. BATE, 2002 Embryonic assembly of a central pattern generator without sensory input. Nature 416: 174-178.

SYKES, P. A., and B. G. CONDRON, 2005 Development and sensitivity to serotonin of Drosophila serotonergic varicosities in the central nervous system. Developmental Biology 286: 207-216.

TAKAHASHI, N., L. L. MINER, I. SORA, H. UJIKE, R. S. REVAY *et al.*, 1997 VMAT2 knockout mice: Heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. Proceedings of the National Academy of Sciences 94: 9938-9943.

TAKAMORI, S., M. HOLT, K. STENIUS, E. A. LEMKE, M. GRONBORG *et al.*, 2006 Molecular Anatomy of a Trafficking Organelle. Cell 127: 831-846.

TAKEMURA, S.-Y., Z. LU and I. A. MEINERTZHAGEN, 2008 Synaptic circuits of the Drosophila optic lobe: The input terminals to the medulla. The Journal of Comparative Neurology 509: 493-513.

TAN, WAITES, LIU, D. E. KRANTZ and R. H. EDWARDS, 1998 A leucine-based motif mediates the endocytosis of vesicular monoamine and acetylcholine transporters. J Biol Chem 28: 17351-17360.

THOMSON, A. M., 2000 Facilitation, augmentation and potentiation at central synapses. Trends in Neurosciences 23: 305-312.

TOOZE, S. A., and W. B. HUTTNER, 1990 Cell-free protein sorting to the regulated and constitutive secretory pathways. Cell 60: 837-847.

TORRES, G. E., R. R. GAINETDINOV and M. G. CARON, 2003 Plasma membrane monoamine transporters: Structure, regulation and function. Nature Reviews Neuroscience 4: 13-25.

TORRI-TARELLI, F., F. GROHOVAZ, R. FESCE and B. CECCARELLI, 1985 Temporal coincidence between synaptic vesicle fusion and quantal secretion of acetylcholine. J. Cell Biol. 101: 1386-1399.

TROWBRIDGE, I. S., J. F. COLLAWN and C. R. HOPKINS, 1993 Signal-dependent membrane protein trafficking in the endocytic pathway. Annu Rev Cell Biol 9: 129-161.

TRUE, J. R., 2003 Insect melanism: the molecules matter. Trends in Ecology & Evolution 18: 640-647.

VAN DER BLIEK, A. M., and E. M. MEYEROWITZ, 1991 Dynamin-like protein encoded by the *Drosophila shibire* gene associated with vesicular traffic. Nature 351: 411-414.

VERLINDEN, H., R. VLEUGELS, E. MARCHAL, L. BADISCO, H.-J. PFLÜGER *et al.*, 2010 The role of octopamine in locusts and other arthropods. Journal of Insect Physiology 56: 854-867.

VERSTREKEN, P., O. KJAERULFF, T. E. LLOYD, R. ATKINSON, Y. ZHOU *et al.*, 2002 Endophilin mutations block clathrin-mediated endocytosis but not neurotransmitter release. Cell 109: 101-112.

VERSTREKEN, P., T.-W. KOH, K. L. SCHULZE, R. G. ZHAI, P. R. HIESINGER *et al.*, 2003 Synaptojanin Is Recruited by Endophilin to Promote Synaptic Vesicle Uncoating. Neuron 40: 733-748.

VOGLMAIER, S. M., K. KAM, H. YANG, D. L. FORTIN, Z. HUA *et al.*, 2006 Distinct Endocytic Pathways Control the Rate and Extent of Synaptic Vesicle Protein Recycling. Neuron 51: 71-84.

VOMEL, M., and C. WEGENER, 2008 Neuroarchitecture of Aminergic Systems in the Larval Ventral Ganglion of *Drosophila melanogaster*. PLoS ONE 3: e1848.

WADDELL, S., 2010 Dopamine reveals neural circuit mechanisms of fly memory. Trends in Neurosciences 33: 457-464.

WAGH, D. A., T. M. RASSE, E. ASAN, A. HOFBAUER, I. SCHWENKERT *et al.*, 2006 Bruchpilot, a Protein with Homology to ELKS/CAST, Is Required for Structural Integrity and Function of Synaptic Active Zones in Drosophila. Neuron 49: 833-844.

WAITES, C. L., A. MEHTA, P. K. TAN, G. THOMAS, R. H. EDWARDS *et al.*, 2001 An Acidic Motif Retains Vesicular Monoamine Transporter 2 on Large Dense Core Vesicles. J. Cell Biol. 152: 1159-1168.

WANG, Y.-M., R. R. GAINETDINOV, F. FUMAGALLI, F. XU, S. R. JONES *et al.*, 1997 Knockout of the Vesicular Monoamine Transporter 2 Gene Results in Neonatal Death and Supersensitivity to Cocaine and Amphetamine. Neuron 19: 1285-1296.

WARZECHA, A. K., M. EGELHAAF and A. BORST, 1993 Neural circuit tuning fly visual interneurons to motion of small objects. I. Dissection of the circuit by pharmacological and photoinactivation techniques. Journal of Neurophysiology 69: 329-339.

WENK, M. R., and P. DE CAMILLI, 2004 Protein-lipid interactions and phosphoinositide metabolism in membrane traffic: Insights from vesicle recycling in nerve terminals. Proceedings of the National Academy of Sciences of the United States of America 101: 8262-8269.

WHEELER, D. A., M. J. HAMBLEN-COYLE, M. S. DUSHAY and J. C. HALL, 1993 Behavior in Light-Dark Cycles of Drosophila Mutants That Are Arrhythmic, Blind, or Both. Journal of Biological Rhythms 8: 67-94.

WICKER-THOMAS, C., and M. HAMANN, 2008 Interaction of dopamine, female pheromones, locomotion and sex behavior in *Drosophila melanogaster*. Journal of Insect Physiology 54: 1423-1431.

WILENS, T., 2006 Mechanism of action of agents used in attention-deficit/hyperactivity disorder. J Clin Psychiatry 67: Suppl 8:32-38.

WILLARD, A., 1981 Effects of serotonin on the generation of the motor program for swimming by the medicinal leech. The Journal of Neuroscience 1: 936-944.

WILSON, N. R., J. KANG, E. V. HUESKE, T. LEUNG, H. VAROQUI *et al.*, 2005 Presynaptic Regulation of Quantal Size by the Vesicular Glutamate Transporter VGLUT1. J. Neurosci. 25: 6221-6234.

WILSON, R. I., and G. LAURENT, 2005 Role of GABAergic Inhibition in Shaping Odor-Evoked Spatiotemporal Patterns in the *Drosophila* Antennal Lobe. J Neurosci 25: 9069-9079.

WOJCIK, S. M., S. KATSURABAYASHI, I. GUILLEMIN, E. FRIAUF, C. ROSENMUND *et al.*, 2006 A Shared Vesicular Carrier Allows Synaptic Corelease of GABA and Glycine. Neuron 50: 575-587.

WOJCIK, S. M., J. S. RHEE, E. HERZOG, A. SIGLER, R. JAHN *et al.*, 2004 An essential role for vesicular glutamate transporter 1 (VGLUT1) in postnatal development and control of quantal size. PNAS 101: 7158-7163.

YAMAMOTO, D., J.-M. JALLON and A. KOMATSU, 1997 Genetic dissection of sexual behavior in *Drosophila Melanogaster*. Annual Review of Entomology 42: 551-585.

YASUYAMA, K., I. A. MEINERTZHAGEN and F.-W. SCHÜRMANN, 2002 Synaptic organization of the mushroom body calyx in Drosophila melanogaster. The Journal of Comparative Neurology 445: 211-226.

YELLMAN, C., H. TAO, B. HE and J. HIRSH, 1997 Conserved and sexually dimorphic behavioral responses to biogenic amines in decapitated Drosophila. Proceedings of the National Academy of Sciences 94: 4131-4136.

YUAN, Q., W. J. JOINER and A. SEHGAL, 2006 A Sleep-Promoting Role for the Drosophila Serotonin Receptor 1A. Current Biology 16: 1051-1062.

YUAN, Q., F. LIN, X. ZHENG and A. SEHGAL, 2005 Serotonin Modulates Circadian Entrainment in Drosophila. Neuron 47: 115-127.

ZHANG, B., Y. H. KOH, R. B. BECKSTEAD, V. BUDNIK, B. GANETZKY *et al.*, 1998 Synaptic Vesicle Size and Number Are Regulated by a Clathrin Adaptor Protein Required for Endocytosis. Neuron 21: 1465-1475.

ZHANG, X., J.-M. BEAULIEU, T. D. SOTNIKOVA, R. R. GAINETDINOV and M. G. CARON, 2004 Tryptophan Hydroxylase-2 Controls Brain Serotonin Synthesis. Science 305: 217-.

ZHOU, C., Y. RAO and Y. RAO, 2008 A subset of octopaminergic neurons are important for Drosophila aggression. Nat Neurosci 11: 1059-1067.

ZHU, Y., A. NERN, S. L. ZIPURSKY and M. A. FRYE, 2009 Peripheral Visual Circuits Functionally Segregate Motion and Phototaxis Behaviors in the Fly. Current biology : CB 19: 613-619.

ZINSMAIER, K. E., A. HOFBAUER, G. HEIMBECK, G. O. PFLUGFELDER, S. BUCHNER *et al.*, 1990 A cysteine-string protein is expressed in retina and brain of Drosophila. J Neurogenetics 7: 15-29.