Development and sensitivity to serotonin of *Drosophila* serotonergic varicosities in the central nervous system

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Abstract

Serotonin is a classical small-molecule neurotransmitter with known effects on developmental processes. Previous studies have shown a developmental role for serotonin in the fly peripheral nervous system. In this study, we show that serotonin can modulate the development of serotonergic varicosities within the fly central nervous system. We have developed a system to examine the development of serotonergic varicosities in the larval CNS. We use this method to describe the normal serotonergic development in the A7 abdominal ganglion. From first to third instar larvae, the volume of the neuropil and number of serotonergic varicosities increase substantially while the varicosity density remains relatively constant. We hypothesize that serotonin is an autoregulator for serotonergic varicosity density. We tested the sensitivity of serotonergic varicosities to serotonin by adding neurotransmitter at various stages to isolated larval ventral nerve cords. Addition of excess exogenous serotonin decreases native varicosity density in older larvae, and these acute effects are reversible. The effects of serotonin appear to be selective for serotonergic varicosities, as dopaminergic and corazonergic varicosities remain qualitatively intact following serotonin application.

**Keywords:** Neurite formation; Neurotransmitter; CNS development

Introduction

Small-molecule neurotransmitters such as serotonin are important modulatory signals in neuronal development (Gaspar et al., 2003). In general, synaptic neuropil is almost always broadly and evenly innervated with serotonergic varicosities such that all regions should receive equal quantities of serotonin (Bunin and Wightman, 1998). Serotonergic varicosities, like those of other neuromodulatory neurons, are thought to engage primarily in volumetric-type neurotransmission in which neurotransmitter is released for distribution over a region of neuropil containing many target synapses (Bunin and Wightman, 1999). As such, serotonergic varicosities often do not have post-synaptic partners. While serotonergic varicosity density should be very important for function and seems to be constant for a given brain region (Oleskevich and Descarries, 1990), it is unclear how serotonergic varicosity formation is spatially regulated. One role for serotonin may be to autoregulate the spacing of neuronal processes. In general, neurons form synapses in a space-filling manner such that volume coverage is maximized (Panico and Sterling, 1995). This process is similar to tiling in sensory dendrites in which the distributive spacing of neuronal processes make receptive fields non-redundant (Jan and Jan, 2003). While a number of molecules are known to regulate these spatially complex processes, it is as yet unclear what the mechanism is (Zinn, 2004).

The neuropil within the fly CNS has distinct anatomical landmarks that can be reproducibly discriminated between samples (Landgraf et al., 2003). Identification of the 84 serotonergic neurons within the fly ventral nerve cord is...
possible due to a number of selective and cell-specific markers and a predictable anatomical distribution (Valles and White, 1988). Serotonin immunoreactivity and serotonin uptake are confined to the serotonergic neurons, which also express the serotonin biosynthesis enzyme dopa decarboxylase (Ddc, involved in serotonin and dopamine synthesis) (Lundell and Hirsh, 1994). The dopaminergic neurons are also distinct from the serotonergic neurons (Budnik et al., 1986; Valles and White, 1986), though the dopaminergic neurites are found in the same general regions as those of the serotonergic system. The regular structure of the fly CNS allows for consistent identification of particular regions of synaptic neuropil from different animals.

Serotonin is known to modulate neuronal branch spacing. The number of serotonergic varicosities in the peripheral nervous system (PNS) of Drosophila doubles when serotonin and dopamine synthesis is reduced (Budnik et al., 1989), suggesting that endogenous serotonin acts to maintain proper neuronal architecture, and that removing this inhibitory influence causes excess branching. The Helisoma serotonergic neuron ENC1 (embryonic neuron C1) increases its branch density when p-chlorophenylalanine (inhibits tryptophan hydroxylase resulting in decreased serotonin synthesis) is administered, while 5-hydroxytryptophan, which increases serotonin levels and decreases the number of branch points (Diefenbach et al., 1995). The response of neuronal processes to serotonin in vitro has been characterized in a number of systems by examining the formation and retraction of growth cones (Torreano et al., 2005; Koert et al., 2001). In some cell types, serotonin inhibits growth cone mobility through activation of voltage-dependent calcium channels (Kater and Mills, 1991). The Helisoma glutamatergic buccal ganglion neuron 19 (B19) demonstrates marked inhibition of its growth cone by serotonin (Haydon et al., 1984); these effects can be mimicked by electrical activity (Cohan and Kater, 1986) and blocked by acetylcholine (McCobb et al., 1988).

We have developed a method to measure the modulatory effects of serotonin on serotonergic neurite spacing in the Drosophila CNS. The native structure of the nerve cord is preserved using a cultured explant strategy, and spinning disk confocal microscopy was used to capture and reconstruct the three-dimensional arrangement of the serotonergic neuropil. As serotonergic neurons develop, serotonin may act as an autocrine/paracrine signal to direct the formation and retraction of varicosities within neuropil to maximize innervation within the tissue and prevent exuberant connections from persisting.

Materials and methods

Fly stocks

The following fly stocks were obtained from Bloomington stock center (http://flystocks.bio.indiana.edu/): CantonS (CS); OregonR (OR); UAS-mCD8-GFP; UAS-syb-GFP; ddc-GAL4; eg me3160; and ddc P-element insertion (y+ w67c23; P[w+ + mC = lacZ]/Ddck02104/CyO). The TH-GAL4 stock was a gift from Jay Hirsh (University of Virginia).

Preparation of ventral nerve cords (VNCs)

Age-matched larval VNCs were dissected in Schneider’s insect media (25°C) and mounted to #1 glass microscope coverslips (18 mm²) in 2 mL fresh media. Serotonin (5-hydroxytryptamine; Sigma, St. Louis, MO) was dissolved in dH2O and diluted 1:1000 with fresh insect media for VNC applications. Tissue fixation was by adding 4 mL of fresh 4% paraformaldehyde for 60 min (3% final PFA concentration), followed by washing with 1× PBS and 1× PBT. Samples were incubated with primary antibodies overnight at 4°C, in 2 mL 1× PBT with 1:667 anti-serotonin (rabbit polyclonal, ImmunoStar) and 1:2000 anti-GFP (3E6 mouse monoclonal, Molecular Probes), except UAS-CD8-GFP; TH-GAL4 where anti-5HT was omitted and CantonS and OregonR, where anti-GFP was omitted. Secondary antibodies (fluorescein-conjugated goat-anti-mouse and rhodamine-conjugated goat-anti-rabbit) were obtained from Jackson Laboratories, and used 1:1000 in 2 mL 1× PBT, overnight at 4°C. All immunostained VNCs were mounted to microscope slides in 90% glycerol/2.5% DABCO (Sigma) and stored at −20°C prior to imaging. The Ab7 left ganglion was imaged from all samples.

Varicosity measurement and visualization

Samples were imaged with a Nikon eclipse E800 microscope (100×, oil-immersion lens, NA = 1.3), Hamamatsu ORCA-ER camera, and a Perkin-Elmer spinning disc confocal unit. The microscope and camera were calibrated using a micron scale (Sigma) and the Focal Check Fluorescent Microspheres Kit (6 μm, Molecular Probes, F-24633). The distal abdominal ganglia were imaged from the cell bodies through the dorsal surface of the neuropil (250–600 optical slices) with 1× 1 binning and 0.094-μm-thick sections. Exposure times varied from 150 to 1000 ms, depending on the intensity of the immunofluorescence (typical exposure ∼300 ms). These parameters over-sample the tissue such that the resolution limit of light microscopy is the limiting factor for visualization. Serial images were auto-leveled in Adobe Photoshop and then imported into Velocity 2.0 for 3-dimensional rendering and varicosity quantification. Each VNC processed generates approximately 1 GB of data and takes a minimum of 1.5 h of computational processing time with a Macintosh PowerPC G4. Varicosities were classified within the anatomical boundaries of the A7 ganglia. To manually measure varicosity volume, varicosities were identified in single confocal slices. Varicosities were defined as swellings in branches in which the swelling was at least twice the
thickness of a branch. To estimate the volume, varicosities were assumed to be ellipsoids with the major axis along the branch, and the first minor axis as the width. The second minor axis, which would be perpendicular to the plane of the photograph, was assumed to be the same as the first minor axis. For manual density measures, varicosities were counted in a single confocal slice of defined area. As varicosities are about 0.5–1.0 μm, it was estimated that each slice represented varicosities in a 1-μm-thick slice. Therefore, the 3D density could be estimated. For each sample, density measures were taken at 2-μm intervals through a sample (about 20 slices) and averaged.

Varicosities and synapses may be functionally distinct. Synapses indicate a region of direct communication between two neuronal processes, with a characteristic profile under electron microscopy, whereas varicosities describe presynaptic swellings that contain a variety of vesicular proteins and neurotransmitter (Ahmari et al., 2000). Previous studies have characterized varicosities as biochemical isolates containing various proportions of synaptic vesicles and mitochondria; while varicosities vary in size, rodent varicosities have been generally characterized as >0.4 μm in diameter, while the intra-varicosity neurite segments are <0.4 μm in diameter (Dori et al., 1998; Shepherd and Harris, 1998).

The signal from the entire intensity distribution was included for analysis, with a size (i.e., volume) inclusion range of 0.2–8 μm³, and Volocity options for noise reduction and object separation were selected. Sample density was calculated by Volocity-counting the number of varicosities within a rectangular solid of known volume from the central region of the neuropil, excluding the gaps at the edges of the neuropil.

The Tukey–Kramer Multiple Comparisons Test and ANOVA analyses were performed for all statistical comparisons using Graphpad InStat 3.0 for Macintosh. These tests evaluate whether the means of three or more independent variables differ. All graphs were constructed using Graphpad Instat for Macintosh and mean ± SD are shown in all figures. For each data point, 6 samples were used.

Results

Serotonergic structure within the ventral nerve cord

The 7th abdominal ganglia (Ab7) of the Drosophila larval ventral nerve cord (VNC) were selected as the anatomical region for all analyses (Campos-Ortega and Hartenstein, 1985). This region has a regular anatomy, and can be consistently identified between sample nerve cords of different developmental stages (Figs. 1A, B). Larval development is divided into three instar phases: first (L1) and second (L2) instar correspond to the first and second days following embryogenesis, respectively, while third instar (L3) begins on the third day after embryogenesis and lasts until pupation (around day 6). Third instar larvae can further be classified on the basis of behavior, with early L3 larvae foraging for food (L3-F, around 72 h post-hatching) and late L3 larvae wandering prior to pupation (L3-W, around 120 h post-hatching). Serotonergic neurons are located in the ventral region of the VNC, and extend neurites into the neuropil (Figs. 1A–D). Dopaminergic neurons are distributed into two main groups: dorsal lateral dopaminergic neurons, which are lateral to the serotonergic neurons, and medial dopaminergic neurons, located ventrally to the serotonergic neurons (Fig. 1B) (Lundell and Hirsh, 1994). In addition, lateral to the serotonergic neurons is a non-serotonergic, ddc-positive corazonergic neuron, which contributes to the central region of neuropil (Fig. 1D, green) (Landgraf et al., 2003). Dopaminergic and corazonergic (i.e., ddc-positive, serotonin-negative) branching in these ganglia begins around second instar (2 days), after serotonergic neurons have established a general region of distribution, and contributes to the central and dorsal varicosities; these processes continue to develop into third instar (Figs. 1C, D). As the larvae mature from L1 to L3, serotonergic neurites in this region increase and the volume of the Ab7 expands while maintaining varicosity density and general architecture (Figs. 1E, F). The volume of the neuropil increases from 5637 ± 794 μm³ at L1 to 2733 ± 1398 μm³ at L3-W (n = 6). Colocalization of serotonin with the GFP labeled synaptic vesicle protein synaptobrevin (syb-GFP, Zhang et al., 2002) is shown for a representative region of serotonergic neurons from first instar UAS-syb-GFP, egm360 AB larvae VNC (Figs. 1G, H). Note that the serotonin-immunoreactive swellings (red) colocalize with synaptobrevin-GFP (green). Non-serotonergic varicosities in Figs. 1G, H are from the corazonergic neuron, which also expresses egGAL4. Syb-GFP puncta are 0.92 ± 0.15 μm in diameter (n = 44), which corresponds to a volume of 0.45 ± 0.23 μm³ assuming these are spheres. This matches well with serotonergic varicosity structure in the vertebrate cortex (Cohen et al., 1995). Note that serotonin immunoreactivity is diffuse throughout the entire cell, filling the soma, nucleus, and fine processes. This finding is consistent with serotonin immunohistochemical staining from grasshopper, and is not likely to be an artifact of the fixation or staining process (Condron, 1999).

Quantifying varicosity density

A method to measure varicosity volume and density was developed. Serotonergic varicosities in the Ab7 region were visually identified as serotonin-immunoreactive swellings larger than the intervening branches in a third instar VNC (Figs. 2A, B). The volume of these swellings was determined manually and by using Volocity 2.0 software; the distribution of varicosity volumes is shown in Fig. 2C. The average varicosity volume for this selection was 0.7 ± 0.2 μm³, and most volumes are between 0.2–8.0 μm³ (assuming a true sphere, this corresponds to an axial diameter
of 0.36–1.24 μm in length). Structures less than 0.2 μm³ typically represent intervening branch structures of the neurites. Note that the limit of resolution using confocal microscopy is about 0.25 μm in length, corresponding to a spherical volume of 0.065 μm³, which is 10-fold smaller than the structures of interest. Varicosity density was calculated from manual identification of varicosities in serial sections of neuropil and compared with computer-assisted density calculations (Fig. 2D). A series of VNCs were repeatedly processed (following an overnight freeze–thaw cycle), and the number of varicosities within the Ab7 region was independently determined (data not shown). The sampling error using this method is 7.1 ± 3.2%, which accounts for errors due to image acquisition, photobleaching, and user-defined selection of the anatomical region of interest as well as computerized classification of varicosities. This error is smaller than the error between age-matched VNCs (Fig. 2E) and is not thought to significantly skew further analyses.

**Varicosity density during development**

Varicosity density measurements from wild-type (wt) larvae were made to establish a normal development curve (Fig. 2E). Varicosities are added throughout the larval period as the VNC increases in size and complexity. The varicosity density remains about the same from L1 to L3-F, but becomes increasingly variable as the larvae approach pupation. There is no significant difference between the isogenic UAS-mCD8-GFP;ddc-GAL4 (doubly homozygous) (Lee and Luo, 1999; Li et al., 2000) and two classic wild-type fly strains (Oregon R and Canton S) during the larval period (Fig. 2E). The UAS-mCD8-GFP;ddc-GAL4 fly line was used as a control for all further studies; this strain expresses membrane-localized GFP in all cells expressing ddc (dopa decarboxylase). In the VNC, the serotonergic, dopaminergic, and corazonergic cells are labeled with mCD8-GFP (Lee and Luo, 1999; Novotny et al., 2002).

**Serotonin levels modulate varicosity formation**

Previous studies have suggested that decreased levels of serotonin increase the number of serotonergic varicosities in the PNS (Budnik et al., 1989). Deficits in dopa decarboxylase function were examined using a mutant with a...
P-element insertion in the ddc gene: homozygous larvae were identified as described (Budnik et al., 1989). These larvae were very slow growing, and at larval day 6, the homozygous mutant resembled a second instar larvae (day 2) in size; these mutants had difficulty moving, and appeared generally unhealthy. The development of the larval cuticle is dependent upon ddc activity, contributing to the gross phenotype of the larvae (Budnik et al., 1989). This severe gross growth phenotype would confound any interpretation of serotonergic varicosity density. All ddc mutant larvae died prior to reaching an equivalent size to day 3 wt larvae (around larval day 7).

In order to further test the role of serotonin on varicosity density, exogenous serotonin was added to intact VNCs in culture to assay the effects on varicosity density at different developmental time points. We systematically characterized the development of wt varicosity density (Figs. 2E and 3A, B), applied exogenous serotonin (Figs. 3C, D), and then removed the exogenous serotonin and allowed for post-treatment recovery (Figs. 3E, F). As a further measure of serotonin selectivity, the effects of serotonin were measured on the dopaminergic neurons only, using the UAS-CD8-GFP/+;TH-GAL4/+ strain, which expresses CD8-GFP in only tyrosine hydroxylase-expressing cells (i.e., dopaminergic neurons) (Friggi-Grelin et al., 2003). Unlike serotonergic varicosities, which exhibit a distinct phenotype upon serotonin application (Fig. 3C), incubation of VNCs with 10 or 100 μM serotonin does not qualitatively appear to decrease dopaminergic architecture in L3-F larvae, but instead allows for an increase in dopaminergic branching in the dorsal region of the neuropil (Figs. 3G, H).

Age-matched randomly sorted UAS-CD8-GFP;ddc-GAL4 VNCs were placed into one of four treatment groups (0, 1, 10, or 100 μM serotonin in insect media), incubated for 3 h (25°C), and processed for serotonin immunofluorescence. Varicosity density was measured for the serotonergic neurons in the usual manner. Serotonergic varicosities in L1 larvae are not responsive to serotonin; L2 and L3-F larval VNCs exhibit a dose-dependent decrease in serotonergic varicosity density as exogenous serotonin is increased from 1 μM to 100 μM serotonin (Fig. 4A). The effects of exogenous serotonin could be seen as early as 30
min following incubation, though no further decrease was observed if incubation continued for 3 h. The changes in varicosity density following exogenous serotonin are visually apparent (compare Figs. 3A, B with Figs. 3C, D), and do not appear to disrupt the general organization of the VNC. Serotonergic varicosities also decrease in volume following treatment with serotonin, from $0.81 \pm 0.11 \mu m^3$ (L3-F control) to $0.73 \pm 0.06 \mu m^3$ with $10 \mu M$ serotonin (ns) and to $0.58 \pm 0.07 \mu m^3$ with $100 \mu M$ serotonin ($P < 0.001$); the distribution curve of varicosity volume does not change its overall shape, but uniformly decreases in peak amplitude as serotonin is increased (data not shown).

The effects of exogenous serotonin on serotonergic varicosities are reversible (Fig. 4B). Age-matched UAS-CD8-GFP;ddc-GAL4 L3-F VNCs were randomly sorted into groups and treated with either 0 or $100 \mu M$ serotonin for 30 min. Then, the 0-μM control and one 100-μM serotonin-treated group were fixed (Figs. 3A–D); the remaining 100-μM serotonin-treated group were washed with fresh insect media to dilute the serotonin to less than 0.1 μM, and then incubated for an additional 10–180 min (25°C) prior to fixation. All samples were processed for serotonin immunofluorescence. The serotonin-treated/wash-out VNCs regained 92% of the pre-treatment varicosity

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Fig. 3. Exogenous serotonin modulates serotonergic varicosity structure. Left panels A, D, and G are serotonin (red) and GFP (green). Middle panels B, E, and H are serotonin alone. Right panels C, F, and I are GFP alone. Panels J and K are GFP alone. The GFP in panels A–I is driven by ddcGAL4 and so labels serotonergic, corazonergic, and dopaminergic neurons. The GFP in panels J and K is driven by TH-GAL4 and labels the dopaminergic neurons. Panels A–C are control non-treated tissue. Panels D–F are after 30 min in 100 μM serotonin. Panels G–I are same as panels D–F except that the serotonin was washed out and samples left sit in media for 30 min. Panel J is an untreated control while panel K was treated in the same way as panels D–F. 5HT-treated dopaminergic branches (K) show a consistent extraneous growth at the dorsal part of the sample. Scale bar = 5 μm.
density following a 30-min recovery (Figs. 3E, F and 4B). In addition, there was a continued increase (above pre-treatment baseline) in varicosity density as the recovery time extended to 3 h (Fig. 4B).

In order to verify that serotonin induces a loss of serotonergic varicosities, a quantifiable GFP marker was used. As ddc-GAL4 induces the expression of GFP in both serotonergic and interspersed dopaminergic varicosities, it is difficult to quantify the loss of serotonergic-GFP alone. Therefore, another driver, egGAL4, was used which induces expression in serotonergic and corazonergic neurons in the L3 CNS (Fig. 5). The corazonergic branches are confined to two small regions of the CNS (green regions in Fig. 4A) and are generally not varicosity-classified as they form optically non-resolved structures greater than the 8 μm³ size cutoff. For density measurements, the midline region of neuropil most populated by corazonergic processes was also excluded from the analysis. Compared to control, serotonin induces a significant loss of both GFP and serotonin. After washout, densities of GFP- and serotonin-labeled varicosities return to normal.

Discussion

These studies indicate that although the CNS varicosity pattern is very complex, with respect to the PNS, the overall density of varicosities is largely regulated as the neuropil increases in size by fivefold. Developing neurites growing in two dimensions interdigitate through a process known as tiling (Jan and Jan, 2003; Wassle et al., 1981), such that each neuron can maximize the non-redundant spatial distribution of a given area. As tiling occurs, many neurons also begin synthesizing their own neurotransmitter (Nguyen et al., 2001). In our study, we have focused upon spacing within three dimensions, using density as a measure of varicosity distribution.

Exogenous serotonin modulates varicosity density

The addition of exogenous serotonin causes a dose-dependent decrease in varicosity density beginning in L2 larvae; however, serotonin has no significant effect on varicosity density for L1 VNCs. These findings suggest that the modulatory effects of serotonin on neuronal structure are temporally regulated, and these sensitivities of older larvae to serotonin may represent a molecular change as larvae develop. Also, all varicosities may not be equally responsive to serotonin. When exogenous serotonin is added in increasing concentrations (Fig. 4A), the decreases in varicosity density in second and third instar larvae appear to settle at the same absolute levels, which might represent set points, despite different initial volumes of neuropil. Despite the known exogenous serotonin concentrations in the media, the concentration of serotonin within the neuropil remains unknown at present, due to unknown diffusion characteristics through the glial barrier of the VNC, as well as unknown degradative and uptake kinetics for serotonin within an intact nervous system. Changes in varicosity density account for most of the effects following manipulation of serotonin levels. Previous studies have utilized the ddc mutant to globally eliminate serotonin (and consequently, dopamine) from the larvae (Budnik et al., 1989). Our evaluation depends upon an ability to stage larvae based upon size and age, and the developmental deficiencies seen in the ddc mutant confirmed our interpretation of varicosity maturation levels in the CNS.

Plasticity in the fly serotonergic system is modulated by serotonin

The acute effects of increased exogenous serotonin are reversible (Fig. 4B). Serotonergic varicosities are eliminated following incubation with serotonin; dilution of serotonin to less than 0.1 μM is permissive for the recovery of serotonergic varicosities to levels comparable with pretreatment controls. Once recovery has occurred, the serotonergic varicosity density remains stable for a prolonged period. These findings indicate the potential for rapid turnover in serotonergic varicosities provided the appropriate stimulus. Live imaging of the effects of serotonin may provide a further method to evaluate the role of transmitter on neuronal structure. The effects of serotonin are selective...
for serotonergic neurons, and serotonin does not appear to disrupt the gross organization of the neuropil. When serotonergic varicosities retracted following exogenous serotonin application in L3-F larvae, dorsal dopaminergic branches appear to increase in complexity; this interaction between the serotonergic and dopaminergic systems remains an interesting area for future studies. In addition, evaluating the pharmacological and genetic basis for dopaminergic varicosity maturation may be possible using the UAS-CD8-GFP;TH-GAL4 strain and the method outlined here for serotonergic neurons.

**Extrasynaptic transmission of serotonin may change neurite structure**

The synaptic cleft concentrations of neurotransmitter following release have been estimated in the rodent at 6 nM serotonin, which is significantly greater than the affinity of the mammalian 5HT1 receptors; however, once serotonin diffuses into the extracellular space, its maximal concentration of 55 nM is close to the receptor affinity and Km for transport (Bunin and Wightman, 1998). Serotonin under these conditions may diffuse more than 20 μm in the rodent.
brain following synaptic release (Bunin and Wightman, 1998). Such high serotonin levels likely facilitate extrasynaptic transmission, as concentration-dependent diffusion away from reuptake sites may allow for local spread and receptor activation. Extrasynaptic volume transmission for dopamine, a neurotransmitter with similar properties to serotonin, shows a maximum transmitter concentration of 2–3 μM up to 100 μm away from the release site; these levels are sufficient to activate all of the dopamine receptor subtypes (Cragg et al., 2001). Those constraints which influence dopamine volume transmission (such as uptake, concentration in terminals and diffusion rates) are similar for serotonin, suggesting that local transmitter release may have a significant effect on local transmitter concentration, and this field of influence may extend a significant distance from the point of release. From the density measure, the average radial spacing of serotonergic varicosities in the L3-W wt larvae is 3.5 ± 0.3 μm. Provided that serotonin is released at concentrations comparable to dopamine, serotonergic varicosities may be influenced by local extrasynaptic transmission. Furthermore, ddc genetic mosaics in the fly result in serotonergic neurons that do not adequately synthesize serotonin; however, local transmitter spread within a segment allows serotonergic neurons to acquire serotonin from the contralateral sib neurons (Valles and White, 1990).

**Varicosity identification accounts for anatomical variation**

The structure of serotonergic neurites undergoes many morphological changes during the larval period. Due to the consistent anatomy of the fly VNC, varicosities from an equivalent region can be measured in different samples, regardless of subtle changes in tissue dimensions as the larvae grow. Since the entire abdominal ganglia can be observed and measured, sampling errors have been estimated to be less than 10% and less significant than the individual variation between age-matched larval VNCs. Colocalization studies of presynaptic proteins and serotonin indicate that the volume distribution of varicosities is within the 0.2–8.0 μm³ range used for Velocity classification, and that these regions are likely to be active zones for transmitter release. Nascent varicosities that have not reached the lower threshold of 0.2 μm³ may be missed by these arbitrary limits, though our estimations are that >90% of all varicosities are appropriately numerated using this process, and any excluded varicosity population would be omitted in every sample.

**Serotonin and varicosity formation**

Changes in serotonin levels rapidly modulate varicosity density within the developing fly CNS. Exposure of serotonergic neurons to serotonin decreases varicosity density in older larvae, while increasing varicosity density in very young larvae, suggesting developmental influences on varicosity formation. Time-lapse imaging of serotonergic neuron-specific markers will resolve whether the effects of serotonin on varicosity density result from changes in addition or retraction rates of varicosities. In addition, serotonin may preferentially effect varicosities of a particular age/stage of development: one hypothesis is that in L3-F larvae, older varicosities are stabilized by activity and insensitive to local serotonin effects, while younger synapses may retract following exposure to serotonin (and/or activity). We predict that decreasing neuronal activity and neurotransmitter release should increase varicosity density in the fly CNS. While inhibition of neurotransmitter release in Munc18-1 mice does not prevent synapse formation or disrupt general morphological organization of the brain, maintenance of neuronal circuits is dependent upon transmitter release (Verhage et al., 2000). The role of neuronal activity has been shown to be developmentally regulated, such that activity enhances axonal filapodial dynamics in young tissues and inhibits motility in older tissues (Tashiro et al., 2003). In addition, neurotransmitter function may induce changes in the surrounding neuropil that may have secondary (feed-back) effects on the developing neuron, through which pre- and post-synaptic terminals may coordinate and optimize their position (Cohen-Cory, 2002).

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