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Serotonergic dystrophy induced by excess serotonin

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Abstract

Administration of certain serotonin-releasing amphetamine derivatives (fenfluramine and/or 3,4-methylenedioxymethamphetamine, MDMA, 'ecstasy') results in dystrophic serotonergic morphology in the mammalian brain. In addition to drug administration, dystrophic serotonergic neurites are also associated with neurodegenerative disorders. We demonstrate here that endogenously elevated serotonin in the *Drosophila* CNS induces aberrant enlarged varicosities, or spheroids, that are morphologically similar to dystrophic mammalian serotonergic fibers. In *Drosophila* these spheroids are specific to serotonergic neurons, distinct from typical varicosities, and form only after prolonged increases in cytoplasmic serotonin. Our results also suggest that serotonin levels during early development determine later sensitivity of spheroid formation to manipulations of the serotonin transporter (SERT). Elevated serotonin also interacts with canonical protein aggregation and autophagic pathways to form spheroids. The data presented here support a model in which excess cytoplasmic neurotransmitter triggers a cell-specific pathway inducing aberrant morphology in fly serotonergic neurons that may be shared in certain mammalian pathologies.

Introduction

The neuromodulator serotonin (5-hydroxytryptamine, 5-HT) is associated with a wide range of physiology and behavior. Evidence suggests that 5-HT levels and partitioning may be key in the modulation of many behaviors. For example, selective serotonin reuptake inhibitors (SSRIs), which inhibit reuptake from the extracellular environment, and monoamine oxidase inhibitors (MAOIs), which inhibit enzymatic 5-HT degradation, are commonly prescribed to treat mood disorders such as anxiety and depression. Accordingly, alterations in serotonin levels have been associated with a variety of complex behaviors and disorders in invertebrate and vertebrate animal models including aggression (Dierick and Greenspan, 2007), sleep (Yuan et al., 2006), and depressive and anxiety-like behaviors (Holmes et al., 2003).

Dystrophic serotonergic morphology has been reported in a number of mammalian studies including those focused on neurodegenerative disease and toxin administration (O'Hearn et al., 1988; Ueda et al., 1998; Molliver and Molliver, 1990; Molliver et al., 1990; Azmitia and

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Nixon, 2008; Liu et al., 2008). The ability of serotonin to affect vast arrays of circuits relies on the combination of the broad spatial distribution of serotonin release sites and the diffusion of serotonin to relatively distant targets (Bunin and Wightman, 1998). Altered distribution, size, and/or function of serotonergic varicosities may severely impact complex functions and behaviors such as cognition and mood state. Previous work provided analyses of the branch architecture and spatial organization of serotonergic neurons and varicosities in the fly larval abdominal CNS (Chen and Condron, 2008; Chen and Condron, 2009), but the cellular mechanisms responsible for this patterning are largely unknown. A number of studies indicate that serotonin itself may play a role in fine-tuning the arborization of serotonergic neurons and the density of serotonin release sites to maintain homeostatic signaling (Whitaker-Azmitia and Azmitia, 1986; Diefenbach et al., 1995; Budnik et al., 1989; Sykes and Condron, 2005). In order to understand how endogenous 5-HT modulates serotonergic morphology we manipulated serotonin levels in *Drosophila* by over-expressing the rate-limiting enzyme in serotonin synthesis, tryptophan hydroxylase (*Trh*). Here we show that increasing serotonin synthesis induces large aberrant swellings, termed 'spheroids,' along serotonergic neurites that are morphologically distinct from normal varicosities. We provide evidence that elevations in cytoplasmic 5-HT trigger this degenerative-like morphological profile that involves ubiquitination and autophagic pathways and propose a novel cellular pathway resulting in dystrophic serotonergic axons.

Results

Prolonged increases in serotonin synthesis specifically in serotonergic neurons leads to reversible spheroid formation

In order to investigate the effects of increased endogenous serotonin production on serotonergic morphology we chose to over-express the rate-limiting enzyme in serotonin synthesis, tryptophan hydroxylase (*Trh*), in serotonergic neurons. Over-expression of *Trh* in monoaminergic neurons results in physiologically relevant increases in 5-HT levels (Yuan et al., 2006; Dierick and Greenspan, 2007) and we confirmed increased serotonin immunoreactivity in larval CNS as assessed by relative pixel intensity (Supp. Fig. 1). Importantly, the overall organization of the serotonergic system is grossly unaffected by *Trh* over-expression. Stereotypical branching patterns (Chen and Condron, 2008) are maintained in the larval abdominal neuropil (not shown) and the overall density of serotonergic varicosities is within the normal range (Supp. Table 1).

When 5-HT synthesis is upregulated, large swellings, or spheroids, are visible along serotonergic immunoreactive neurites in larval and adult fly neuropil occurring both *en passant* and as terminal bulbs (Fig. 1A, Supp. Fig. 2). Swellings of this size have never been observed previously despite detailed analyses of larval serotonergic morphology including the over-expression of numerous proteins (Sykes and Condron, 2005; Chen and Condron, 2008; Chen and Condron, 2009). Notably, over-expression of *Trh* does not result in spheroids when targeted to other cell types, including the dopaminergic neurons, which can be induced to synthesize serotonin (Fig. 1C-D). Driving UAS-*Trh* in the serotonergic and dopaminergic neurons simultaneously with *th*-GAL4 (Friggi-Grelín et al., 2003) and *Trh*-GAL4 reveals comparable 5-HT immunoreactivity in both cell types and 5-HT immunoreactive spheroids in the neuropil (asterisks in Fig. 1C). Conversely, expression of UAS-*Trh* in the dopaminergic neurons only also results in 5-HT immunoreactivity comparable to or greater than the serotonergic neurons but a complete lack of 5-HT immunoreactive spheroids in the neuropil (Fig. 1D). We also expressed UAS-*Trh* in the non-monoaminergic *even-skipped* expressing neurons (motor neurons *RN-2*-GAL4, sensory neurons *109(2)80*-GAL4) to test for non-specific effects of construct expression on neuronal morphology and saw no effect (Supp. Fig. 3). All of the GAL4 drivers employed drive membrane-associated GFP at comparable or greater levels

than *Trh*-GAL4 (not shown). Finally, over-expression of a protein with similar function, tyrosine hydroxylase (UAS-TH, True et al., 1999), the rate-limiting enzyme in dopamine synthesis, does not induce aberrant morphology in serotonergic or dopaminergic neurons (Supp. Fig. 3). Manipulations performed to assess specificity are summarized in Table 1. We interpret these negative results as evidence that the structural abnormalities we observe are specific to *Trh*-overexpression in serotonergic neurons.

Varicosities of *Trh* over-expressing serotonergic neurons exhibit significantly decreased volume compared to controls, while spheroids are 10-20 times more voluminous than varicosities, making them easily discernable (Fig. 2A). The number of spheroids in the larval abdominal serotonergic neuropil 72 hours after egg lay (AEL) was assessed and found to be dose-dependent upon transgene expression, although driving UAS-*Trh* with *Trh*-GAL4 always results in spheroids (Fig. 2B). Despite the fact that in wild-type flies *Trh* expression begins 14-18 hours AEL (Neckameyer et al., 2007) and serotonin synthesis begins 16-18 hours AEL (Vallés and White, 1988), serotonergic spheroids are never observed until nearly 48 hours AEL, even when UAS-*Trh* is maximally driven in the serotonergic neurons (Fig. 2C). After spheroids were first observed at 48 hours AEL, there was no effect of age on spheroid number (Fig. 2C, $p = 0.12$, Kruskal-Wallis ANOVA). The delay in spheroid formation following UAS-*Trh* expression could be due to a developmental influence or a temporal lag between the increase in 5-HT and spheroid formation. To address this question, and also whether the structures are reversible, we used a temperature-sensitive *Tubulin*-GAL80 (*Tub*-GAL80^{ts}) to restrict GAL4 activity until first larval instar. *Trh* over-expression was induced with a temperature shift and 5-HT immunoreactive spheroids in the abdominal neuropil were counted. 24 hours after *Trh* over-expression began, 5-HT immunoreactivity was observed in dopaminergic neurons, indicating excess serotonin being taken up by these cells presumably through dopamine transporter (dDAT) activity (Supp. fig. 4). dDAT transport of 5-HT is relatively unfavorable *in vitro* (Pörzgen et al., 2001), however, uptake and storage of 5-HT into dopaminergic neurons via DAT has been repeatedly demonstrated in mammals in cases of elevated extracellular serotonin (for review, Daws, 2009). Spheroids were not observed in serotonergic neuropil until 48 hours after *Trh* over-expression began (Fig. 2D, Supp. Fig. 4), similar to the developmental time-course observed in the absence of a temperature-sensitive element (Fig. 2C). After spheroids formed, larvae were shifted back to the permissive temperature for *Tub*-GAL80^{ts} to inhibit *Trh* over-expression. 24 and 48 hours after GAL4 suppression the number of serotonergic spheroids was significantly less than in temperature matched controls (asterisks compare squares to age-matched circles) (Fig. 2D). Within groups containing GAL80^{ts}, 24 hours of GAL4 suppression significantly reduced the number of spheroids (crosses compare squares to 96 hour time-point within same group), although after 48 hours the differences were not significant (Fig. 2D). This may reflect a lack of full GAL4 suppression by GAL80 or a buildup of earlier serotonin production. Taken as a whole, our data indicate that serotonergic spheroids can be induced after 48 hours of *Trh* over-expression in the mature larva and spheroid formation is reversible upon inhibition of *Trh* over-expression.

Serotonergic spheroids exhibit unusual protein localization

High-resolution confocal imaging of individual serotonergic spheroids revealed atypical localization of serotonergic markers. Normal serotonergic varicosities contain a broad distribution of serotonin immunoreactivity as well as synaptic markers throughout the entire varicosity (Sykes and Condron, 2005; Chen and Condron, 2008; Chen and Condron, 2009; Fig. 1). We used a number of cellular markers to assess protein localization within spheroids (Fig. 3). Protein localization was quantified by obtaining thin confocal sections through the approximate center of spheroids along the Z-axis and measuring pixel intensity along a line placed across the approximate center of the spheroid perpendicular to the Z-axis (Fig. 3 A', B', C,D). Data are presented as the ratio of pixel intensity at the core of the spheroid to the

periphery such that markers giving ratios less than one are preferentially localized to the periphery of the spheroid while markers with ratios greater than one are preferentially localized to the core (Fig. 3E). The serotonergic markers 5-HT, Trh and dSERT, as well as synaptic protein (synaptotagmin-GFP, syt-GFP) and membrane protein (mCD8-GFP), localize to the periphery of spheroids (Fig. 3E). However, soluble GFP as well as autophagy markers GFP-Atg5 and GFP-LC3 (not shown) (Rusten et al., 2004), and human tau protein are present in the spheroid core (Fig. 3E) indicating that the spheroid core is immunohistochemically accessible. The exclusion of normal serotonergic markers from the spheroid core, along with the inclusion of Atg5 and h-tau suggest that these structures are aberrant and relevant to neurodegenerative changes.

Directed screen probing cellular mechanism

Dystrophic serotonergic morphology similar to that presented here has been documented in several pathological conditions including increased oxidative stress (Ueda et al., 1998), neurodegenerative disease and disease models (Azmitia and Nixon, 2008; Liu et al., 2008), and following administration of the serotonergic toxins fenfluramine (one component of the no longer available anorectic drug Fen-phen) and MDMA (O'Hearn et al., 1988; Molliver and Molliver, 1990; Molliver et al., 1990). Based on previous documentation of serotonergic morphological abnormalities, we performed a directed pharmacological and genetic screen to investigate the cellular mechanisms responsible for serotonergic spheroid formation in the fly under the condition of enhanced 5-HT synthesis. In order to observe both increases and decreases in spheroid number we chose to employ a sensitized genetic background with heterozygous UAS-Trh for manipulations probing cellular mechanisms (Fig. 2B, UAS-Trh X1)). We tested increased oxidative stress, altered serotonin synthesis and partitioning, and canonical neurodegenerative pathways as putative mechanisms resulting in spheroid formation. Feeding larvae compounds previously utilized to probe reactive oxygen species (ROS)-related pathways in the fly (Bonilla et al., 2006; Dias-Santagata et al., 2007) failed to affect spheroid number in the sensitized background (Supp. Fig. 5) and therefore we conclude that alterations in ROS formation are not the driving factor in UAS-Trh-induced spheroid formation.

Elevations in cytoplasmic serotonin are responsible for spheroid formation

As Trh is the rate-limiting enzyme in serotonin synthesis, serotonergic spheroids may form as a consequence of increased serotonin production. Inhibition of Trh activity with the competitive serotonin synthesis inhibitor parachlorophenylalanine (PCPA) (Coleman and Neckameyer, 2005) significantly reduced spheroid frequency in serotonergic neuropil (Fig. 4A). PCPA feeding can also inhibit larval dopamine synthesis (Dasari and Cooper, 2007) therefore we employed a second Trh inhibitor, α -methyltryptophan (α -MTP) (Coleman and Neckameyer, 2005; Dierick and Greenspan, 2007), to confirm that excess Trh activity is necessary for spheroid formation (Fig. 4A). We next attempted to indicate the sub-cellular location of serotonin action in spheroid formation by altering partitioning of serotonin in and around the cell by pharmacologically blocking the serotonin plasma membrane and vesicle membrane transporters. Blockade of the cocaine-sensitive plasma membrane serotonin transporter (SERT) by feeding larvae cocaine had no effect on spheroid number (Fig. 4A). However, inhibiting the activity of the vesicle transporter (VMAT) responsible for serotonin uptake and storage in synaptic vesicles using the compound reserpine resulted in a significant increase in the number of serotonergic spheroids (Fig. 4A). We also genetically over-expressed both *dSERT* and *dVMAT* along with *Trh* to assess effects of increases in serotonin transport on spheroid formation and no change in spheroid number was observed (Fig. 4A).

SERT is a major regulator of serotonin localization and if serotonin itself were responsible for spheroid formation it would be surprising that SERT over-expression would not affect spheroid

number. Early manipulations of SERT may have lasting effects on the organism due to the developmental importance of serotonin (Ansorge et al., 2004) and therefore we hypothesized that early compensation was masking effects on serotonin partitioning by SERT. In order to examine the effect of *dSERT* over-expression on spheroid formation we again used *Tub-GAL80^{ts}* to restrict GAL4 activity until first larval instar, bypassing embryonic development. When UAS-Trh and UAS-GFP-dSERT were co-expressed in serotonergic neurons using this paradigm we observed a significant increase in spheroid number that was inhibited by cocaine feeding (Fig. 4B). Furthermore, while cocaine feeding alone had no effect on spheroid number in controls over-expressing *Trh* during early development (Fig. 4A), when *Trh* over-expression is induced later, cocaine completely suppresses spheroid formation (Fig. 4B). These data indicate a potential developmental window during which serotonergic neurons may adapt to excess serotonin. Reserpine feeding enhances spheroid number regardless of early or late *Trh* over-expression except when dVMAT is concurrently over-expressed, presumably indicating that dVMAT over-expression and blockade can counteract one another in spheroid formation (Fig. 4B, dark gray bars). Enhanced 5-HT reuptake and VMAT blockade with reserpine both increase cytoplasmic serotonin concentration, therefore the data presented here support the hypothesis that increases in cytoplasmic serotonin levels drive spheroid formation.

Aggregation inhibitors suppress spheroid formation

The spheroids observed here are reminiscent of degenerating axons seen after injury or in disease states. As similar structures are observed in serotonergic neurons in diseased human brain (Azmitia and Nixon, 2008) and mouse models of amyloid pathology (Liu et al., 2008) we looked for genetic interactions between *Trh* over-expression and human tau and amyloid precursor proteins (APP), which have both been utilized in fly models of neurodegenerative disease (Greeve et al., 2004). Expression of these constructs in serotonergic neurons does not induce serotonergic spheroids in the absence of increased serotonin synthesis nor is serotonergic branch morphology altered (not shown). Coexpression of both h-APP and h-tau (mutant and wild-type) along with UAS-Trh significantly enhanced the number of spheroids in serotonergic neuropil (Fig. 5A). This data taken along with the inclusion of h-tau in the spheroid core (Fig. 3) suggests that aggregation-prone proteins and/or their metabolites may be non-specifically incorporated into spheroids.

Growing evidence indicates that aggregate formation in neurodegenerative disease models may be due to defects in or overwhelming of the ubiquitin-proteasome system (UPS) and autophagic clearance machinery in the cell (Martinez-Vincente and Cuervo, 2007; Tai and Schuman, 2008). The presence of ubiquitin is a common feature of protein aggregates and inclusion bodies in neurodegenerative disease, presumably because misfolded or damaged proteins within the aggregate have been tagged for degradation. The UPS is also required for local degeneration during developmental pruning and following axonal injury in *Drosophila* and mammals (Zhai et al., 2003; Watts et al., 2003). Inhibition of the UPS with a yeast ubiquitin protease (UBP2) was able to suppress spheroid formation almost entirely (Fig. 5A). We observed punctate ubiquitin immunoreactivity within serotonergic spheroids (Fig. 5B), but did not find drastically increased ubiquitin deposits in spheroids when compared to the general neuropil.

As cytological analysis indicated that GFP-Atg5 localized to the spheroid core (Fig. 3E), we tested the ability of autophagy induction and suppression to affect serotonergic spheroid formation. Direct activation of autophagy in serotonergic neurons over-expressing *Trh* using UAS-Atg1 (Scott et al., 2007) resulted in a significant decrease in spheroid formation (Fig. 5A). Conversely, genetically suppressing autophagy by over-expressing Rheb, a positive regulator of TOR (target of rapamycin) (Saucedo et al., 2003), increased spheroid number (Fig. 5A). Pharmacological induction of autophagy with rapamycin (a TOR inhibitor) and lithium

chloride (LiCl), which inhibits inositol monophosphatase and lowers inositol and IP₃ levels, reduces cellular toxicity associated with aggregate formation in *Drosophila* (Berger et al., 2006; Sarkar et al., 2008). We fed *Trh* over-expressing larvae rapamycin, LiCl, and a combination of the two, based on a report that combinatorial treatment may be more effective than either alone in inducing autophagic clearance mechanisms (Sarkar et al., 2008). Both rapamycin and LiCl feeding significantly suppressed spheroid formation, as did the combination of the two with the combinatorial treatment being slightly more effective than LiCl alone (Fig. 5A).

We also tested the effect of feeding cystamine, a transglutaminase (TG) inhibitor, on serotonergic spheroid number. TG is an enzyme responsible for protein crosslinking throughout the body and is a proposed target for treatment of neurodegenerative diseases associated with protein aggregate formation (Wilhelmus et al., 2008). Cystamine has been utilized to reduce toxicity in cell culture, fly and mouse aggregate-forming disease models (Dedeoglu et al., 2002; Apostol et al., 2003; Agrawal et al., 2005). Of particular note to this study, TG is also responsible for crosslinking serotonin to small GTPases in activated platelets, rendering them constitutively active (Walther et al., 2003). Feeding cystamine to larvae over-expressing *Trh* drastically reduced serotonergic spheroid number when compared to controls (Fig. 5A).

Fly spheroids may be analogous to drug-induced swellings along mouse serotonergic fibers

Dystrophic serotonergic axons have been documented in the brains of mice exposed to the serotonin releasing drugs MDMA and fenfluramine (O'Hearn et al., 1988; Molliver and Molliver, 1990). Both drugs interfere with transport at the vesicle and plasma membranes (Rudnick and Wall, 1992; Schuldiner et al., 1993). We attempted to replicate the dystrophic serotonergic phenotype in mammals by treating 3 male C57B1/6J mice with 25mg/kg fenfluramine or vehicle control. Serotonergic axonal morphology in the frontal cortex was examined in animals sacrificed four days after the last injection. As expected, fenfluramine treated mice developed aberrant serotonergic fibers in the forebrain not seen in saline injected controls (Fig. 6A and B). Similar to fly serotonergic spheroids, the axonal swellings culminated in terminal bulbs (Fig. 6C) or appeared along a continuous branch (Fig. 6D). However, the range of serotonergic varicosity volumes in fenfluramine treated mouse brain and saline-injected controls were not significantly different (saline: 0.021-3.06 μm^3 ; fenfluramine: 0.021-2.75 μm^3 , $p = 0.41$, Student's *t*). The axonal swellings ranged from 5-70 μm^3 (Fig. 6E). In mice treated with fenfluramine the density of serotonergic varicosities in frontal cortex was slightly decreased compared to controls (Supp. Table 1). This finding has been previously reported (Appel et al., 1989), however, as fenfluramine is a known 5-HT releaser (Schuldiner et al., 1993) calculation of serotonergic innervation density based on 5-HT immunoreactivity following fenfluramine treatment is likely to be an underestimation.

Discussion

The data presented here characterize a novel structural aberration in serotonergic neurons of *Drosophila*. Increasing serotonin levels by over-expressing the rate-limiting enzyme in serotonin production, *Trh*, in the fly leads to formation of neuritic spheroids similar to axonal spheroids observed during axonal degeneration. Our data indicates that the trigger leading to spheroid formation in these cells is an increase in cytoplasmic serotonin that leads to ubiquitination and autophagic hindrance. The spheroids described here can be blocked pharmacologically by suppressing TRH activity or feeding aggregation inhibitors.

Serotonergic neurons are particularly susceptible to serotonin-induced morphological aberrations in this study. A gene dose-dependent effect on spheroid number was observed in serotonergic neurons (Fig. 2B) and therefore the specificity we observed could be explained

by strength of the GAL4 drivers employed. However, driving UAS-Trh in dopaminergic neurons induces 5-HT immunoreactivity in these cells comparable to that in serotonergic neurons without spheroid formation (Fig. 1C, D) and the non-monoaminergic drivers utilized drive membrane associated GFP expression much more strongly than *Trh*-GAL4 (not shown). Thus, it is likely that some unique properties of serotonergic neurons render them susceptible to the morphological effects of excess 5-HT production in this study. A number of studies have suggested an autoregulatory role for serotonin in serotonergic morphology (Whitaker-Azmitia and Azmitia, 1986; Diefenbach et al., 1995; Budnik et al., 1989; Sykes and Condron, 2005). Larval *Drosophila* ventral nerve cords in explant culture exhibit decreased serotonergic varicosity density and varicosity volume in response to exogenously applied serotonin (Sykes and Condron, 2005). In the current study, upregulating serotonin synthesis did result in decreased serotonergic varicosity volume but there were no discernable effects on varicosity density or overall branch structure. While *Trh* over-expression can increase serotonin synthesis, the impact on serotonin release is unclear and autoregulatory varicosity retraction may require a different regime. The persistent over-expression of *Trh* may allow for adaptation within the system resulting in normal varicosity densities as compared with acute administration of exogenous 5-HT.

We have adopted a model whereby increased serotonin in the cytoplasm induces spheroids along serotonergic neurites. However, a hypothesis that alterations in extracellular 5-HT concentrations via neurotransmitter release are feeding back on serotonergic morphology, either directly or indirectly, could also be supported. We interpreted the increases in spheroid number following VMAT blockade and SERT over-expression as indicative of downstream processes resulting from 5-HT trapped in the cytoplasm, but both of these manipulations also reduce the amount of 5-HT available for extracellular signaling. Similarly, the ability of cocaine to inhibit spheroid formation could reflect a rescue of serotonin signaling. However, if reduced serotonin signaling were responsible for spheroid formation one would expect *Trh* inhibition to exacerbate rather than suppress spheroid formation. Evaluation of the effects of *Trh* over-expression on serotonin release will assist in the interpretation of this data and these studies are currently underway utilizing recently developed techniques for the quantification of 5-HT release in the fly larval CNS (Borue et al., 2009).

Despite the major role of SERT in serotonin partitioning, we observed no effect of dSERT over-expression or cocaine administration on spheroid number unless serotonin levels were elevated after embryonic development (Fig. 4B). Developmental and mature manipulations of SERT activity can have opposing effects on adult behaviors, presumably by altering homeostasis of serotonergic signaling. For example, SERT knockout mice exhibit depressive-like behaviors as adults (Holmes et al., 2003), while administration of selective serotonin reuptake inhibitors (SSRIs) or SERT siRNA to adult mice can actually reduce depressive-like behaviors (Thakker et al., 2005). In the present study, increased serotonin synthesis during embryonic stages may sensitize the system accordingly such that manipulations of the serotonin transporter are ineffective in altering morphology. Our observations are consistent with previous work suggesting that enhancing monoaminergic release by constitutively over-expressing *dVMAT* reduces cocaine sensitivity in adult flies potentially by inducing adaptive mechanisms at monoaminergic release sites (Chang et al., 2006). The later induction of *Trh* over-expression achieved using *Tub*-GAL80^{ts} may leave the serotonergic system maladapted to a relatively sudden upregulation in serotonin levels, allowing SERT manipulations to have more apparent structural effects. The relationship between developmental alterations in serotonin signaling and serotonergic homeostasis in the mature animal is likely a key component to understanding the function and dysfunction of serotonergic signaling and is the focus of future work.

Co-expression of human disease associated proteins such as tau and APP enhance serotonergic spheroid number (Fig. 5A) suggesting that increases in serotonin levels interact with canonical protein aggregation pathways to form swellings. Alternatively, inhibition of the UPS with UAS-UBP2 is able to suppress spheroid formation indicating that protein dysfunction is upstream of spheroid formation. The UPS is required for normal axonal degeneration during *Drosophila* metamorphosis (Watts et al., 2003) and UPS inhibition delays Wallerian degeneration of transected axons (Zhai et al., 2003). There is also evidence that UPS inhibition can induce autophagy to reduce endoplasmic reticulum stress resulting from excess misfolded proteins (Ding et al., 2007). Autophagic mechanisms are now recognized as major contributors to growth restriction in conditions of starvation, programmed cell death (Scott et al., 2007), and axonal degeneration (Wang et al., 2006). When autophagic pathways become overwhelmed by excess misfolded or dysfunctional protein, material may build up to form protein masses or aggregates (Martinez-Vincente and Cuervo, 2007). Our results suggest that upregulation of autophagic pathways can reduce the occurrence of serotonergic spheroids. The concomitant decrease in spheroid number observed after cessation of Trh over-expression may also reflect cellular housekeeping mechanisms (Fig. 2D). A conditional model of Huntington's disease in mice demonstrates that inclusions can be cleared in the absence of other manipulations if the underlying offender (in this case, a mutant huntingtin fragment) is removed (Yamamoto et al., 2000). In the current study, UPS inhibition may indirectly suppress spheroid formation by activating these pathways and further investigation is required to determine the respective roles of the UPS and autophagy in aberrant serotonergic morphology. How cytoplasmic serotonin may interact with the UPS and autophagic pathways, directly or indirectly, is unclear. However, it is of note that low doses of methamphetamine, which increases cytoplasmic concentrations of neurotransmitter in dopaminergic neurons, cause rapid upregulation of autophagy in cell culture (Castino et al., 2008). It remains unknown whether defects in the autophagic pathway cause morphological damage or if cellular damage triggers autophagy induction for cellular reorganization and restructuring.

We replicated serotonergic axonal dystrophy in mice by administering the substituted amphetamine fenfluramine in order to compare quantitative characteristics to our fly model because fenfluramine and MDMA- induced serotonergic axonal dystrophy in mammals shares several characteristics with the fly spheroids reported here: (1) despite damage to serotonergic axons, the cell bodies remain intact (O'Hearn et al., 1988; Appel et al., 1989; Molliver and Molliver, 1990; Fig 1C, Supp. fig. 2), (2) dystrophic axons contain swellings that are on average 5-20 times larger in volume than normal varicosities (Molliver and Molliver, 1990; Fig. 2A, Fig. 6E), (3) axonal damage is observed only after at least 36 hours after drug administration (Molliver and Molliver, 1990) or *Trh* over-expression begins (Fig. 2 C,D), (4) serotonergic damage is suppressed by pharmacological inhibition of the plasma membrane serotonin transporter (Sanchez et al., 2001; Fig. 4B). These drugs of abuse cause mispartitioning of serotonin in and around the cell and could induce damage through yet-unidentified serotonin-dependent pathways. While it is currently unclear whether comparable cellular mechanisms are responsible for serotonergic dystrophy in drug-treated mammals and flies producing excess 5-HT, the morphological similarities are provocative and a focus of future study.

Cytoplasmic serotonin is physically associated with a variety of cellular proteins with functional consequences. In a process known as serotonylation serotonin covalently bound to small GTPases by TG renders these G proteins constitutively active in platelets (Walther et al., 2003), smooth muscle cells (Guilluy et al., 2007), and cultured neurons (Dai et al., 2008). Therefore it is possible that the trigger leading to spheroid formation in fly serotonergic neurons depends upon specific interactions of free serotonin with cellular proteins. Feeding the TG inhibitor cystamine suppressed spheroid formation in our model of excess serotonin production (Fig. 5A). One transglutaminase (*Tg*) gene has been identified in *Drosophila* (CG7356) that appears to be expressed predominantly in cardiac tissue (Iklé et al., 2008) but whether this

functions in the CNS remains unknown. It is tempting to hypothesize that inhibiting TG activity under conditions of excess 5-HT prevents neurotransmitter binding to cytoplasmic proteins that would initiate spheroid formation. However, the specific pathway affected is unclear since TG is a proposed therapeutic target for treatment of general protein aggregation in neurodegenerative disease (Wilhelmus et al., 2008). Measures of cytoplasmic dopamine concentrations in primary neuronal culture recently demonstrated cellular toxicity stemming from elevated cytoplasmic dopamine via metabolite interactions with α -synuclein (Mosharov et al., 2009). It is unknown whether analogous processes occur in serotonergic neurons and future work will attempt to elucidate potential cytoplasmic serotonin targets in the fly CNS.

Experimental methods

Drosophila strains

Unless otherwise noted, all UAS-constructs were expressed under control of the *Trh*-GAL4 driver that specifically drives GAL4 expression in the serotonergic neurons (CG9122). This driver is also known as '*tph*-GAL4' (Chen and Condron, 2008; Borue et al., 2009; Chen and Condron, 2009) and was provided by Jaeseob Kim, (Korea Advanced Institute of Science and Technology). UAS-Trh (Yuan et al., 2006) was provided by Amita Sehgal (UPenn). UAS-TH and UAS-DDC (True et al., 1999) were provided by John True (Stony Brook University). *th*-GAL4 (Friggi-Grelín et al., 2003) was kindly provided by Jay Hirsh (University of Virginia). UAS-hTau and UAS-hTau-R406W (Wittman et al., 2001) were gifts from Mel Feany (Harvard University). UAS-GFP-dSERT⁴ was generated by Tim Lebestky (California Institute of Technology). UAS-Atg1^{6B} (Scott et al., 2007) was provided by Thomas Neufeld (University of Minnesota). UAS-dVMAT-A (Chang et al., 2006) was provided by David Krantz (University of California, Los Angeles).

All other fly strains were obtained from the Bloomington Drosophila Stock Center at Indiana University, Bloomington, IN.

Fenfluramine injections—Animals were housed with ad libitum access to food and water, maintained on a 12-hour light/dark cycle, and under controlled temperature and humidity. All experiments were performed in accordance with the University of Virginia Animal Care and Use Committee. Male C57Bl/6J mice (Jackson, Bar Harbor, Maine) at 6-8 weeks of age received either fenfluramine or saline (n = 3 mice per group). Fenfluramine injections were performed as previously described (Itzhak et al., 2003). d,l-fenfluramine (obtained from the NIDA Drug Supply Program, Rockville, MD) was dissolved in normal saline at a concentration of 2.5mg/mL and administered via intraperitoneal injection at 0.1ml/10g animal weight to achieve a dose of 25 mg/kg. Four injections were spaced 12 hours apart and mice were sacrificed 4 days after the last injection (Itzhak et al., 2003). Mice were anesthetized with a lethal dose of pentobarbital and transcardially perfused at room temperature with 10 ml phosphate buffered saline (PBS) followed by 10 ml of PBS/4% paraformaldehyde over a period of 3-5 minutes. Brains were removed and allowed to fix for 24 hours at 4°C. After equilibrating in 30% sucrose for 48 hours, 40 μ m free-floating coronal cryosections were collected using a sliding cryotome.

Antibodies and immunohistochemistry—*Drosophila* dissections and immunohistochemistry were performed as previously described (Daubert and Condron, 2007). 5-HT immunohistochemistry of free floating mouse sections were performed similarly. The following antibodies were used in this study: rabbit anti-5-HT (1:1000, Immunostar), chicken anti-GFP (1:1000, Aves Labs), mouse 5A6 (anti-human tau, DSHB, 1:100), rabbit anti-Trh (Neckameyer et al., 2007) (1:2000), rat anti-5-HT (1:500, Accurate Chemical), rabbit

monoclonal anti-ubiquitin (Epitomics, 1:500), anti-mouse AlexaFluors 488 and 568, anti-rabbit AlexaFluors 488 and 568, anti-chicken AlexaFluor 488 (1:1000, Molecular Probes).

dSERT polyclonal antibody generation—A synthetic peptide conjugated to KLH was generated corresponding to amino acids 382-396 of dSERT (KTSIDKVGLEGPGI) and used as immunogen for antisera creation in rabbit (Wuhan Lobogene Technology, Ltd). Antisera was subsequently affinity purified and final antibody concentration determined to be 1.13mg/mL (Covance). Polyclonal rabbit anti-dSERT antibody was used at 1:2000.

Pharmacology—A sensitized genetic background with genotype *Trh-GAL4/Trh-GAL4;UAS-Trh/UAS-mCD8::GFP* was utilized for all drug treatments. Early first instar larvae (24 hours) were transferred to 1:1 yeast:water paste containing the appropriate dilution of compound and dissected 48 hours later. Drug concentrations were determined empirically using dilution series. Parachlorophenylalanine (pCPA, 5mg/mL), α -methyltryptophan (α -MTP, 5mg/mL), reserpine (30 μ g/mL), cystamine (0.2mg/mL), rapamycin (1 μ g/mL), lithium chloride (LiCl, 0.2mg/mL), cocaine-HCl (1mg/mL), rotenone, paraquat, melatonin and α -tocopherol (vitamin E) were obtained from Sigma-Aldrich, St. Louis, MO.

Gal80 control of transgene expression—Flies containing a temperature sensitive tubulin-Gal80 construct were crossed into sensitized strains. *TubGal80^{ts}* is inactive at the restrictive temperature (18°C) and is activated at the permissive temperature (30°C). Flies were allowed to develop at the restrictive temperature for 48 hours at which time early first instar larvae were transferred to the permissive temperature and dissected at the indicated time-points. For recovery experiment, larvae were transferred back to the restrictive temperature and dissected at indicated time-points.

Imaging—For mouse sections, coronal sections at the level of 0.65mm rostral to Bregma were photographed. For all pictures, images were obtained using a Nikon eclipse E800 confocal microscope and recorded using Perkin-Elmer software. Images were auto-leveled in Adobe Photoshop.

Quantification of spheroids—A Z-series of the abdominal region of larval CNS stained with a 5-HT antibody was taken at 40X magnification. Confocal stacks were imported into ImageJ and swellings that could be identified as roughly 10 times larger than varicosities were counted between the axonal crossing at segment A1 and the most posterior region of the ventral nerve cord. In circumstances where treatment compromised 5-HT immunoreactivity (i.e. reserpine feeding) spheroids were identified using GFP immunoreactivity.

Volume measurements—Confocal Z-stacks of third instar larval abdominal CNS and mouse sections stained with an antibody against 5-HT were imported into Volocity 3.0 (Improvision, Perkin Elmer, Waltham, Massachusetts, USA), autoleveled and 3-dimensional reconstructions of serotonergic neuropil were created. Volocity software was used to identify individual varicosities (*Trh-GAL4;UAS-mCD8::GFP* flies, saline injected mice) and spheroids (*Trh-GAL4;UAS-Trh* flies, fenfluramine injected mice) and measure their volume. The volume cut-off for differentiating between normal varicosities and spheroids was 8 μ m³ for *Drosophila* and 5 μ m³ for mouse.

Staining intensity analyses—To quantify protein localization within spheroids at confocal microscopy resolution single plane images were obtained of individual spheroids at the approximate center along the z-axis. Pixel intensity along a line across the center of the spheroid was measured in ImageJ. To quantify 5-HT staining intensity the entire abdominal CNS was imaged from the dorsal to the ventral region with exposure time of 200ms at 1 $\times$ 1 binning and

in 0.2 μ m steps. Z-stack compressions were imported into ImageJ and pixel intensity was measured within a rectangle stretching from the serotonergic axon crossing at A6 to the serotonergic axon crossing at A1.

Density measurements—Densities of serotonergic varicosities in the fly were performed as previously described (Sykes and Condron, 2005). Estimation of density of serotonergic spheroids in the fly was performed by taking the average number of spheroids observed in the sensitized background divided by the estimated total volume of the larval abdominal CNS (400,000 μ m³). Densities in the mouse were estimated by dividing the total number of 5-HT immunoreactive varicosities or spheroids observed by the volume of tissue analyzed.

Data analysis—Ordinary and non-parametric Kruskal-Wallis ANOVA were performed using GraphPad InStat software. Student's t tests were performed using Microsoft Excel.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Genetically enhancing 5-HT synthesis in serotonergic neurons induces spheroid formation along 5-HT neurites. (A) All panels show serotonergic neuropil stained with an antibody against serotonin. Serotonergic cell bodies have been optically sectioned from the pictures in VNC panels. (Scale bar = 10 μ m). Over-expression of Trh under the control of the *Trh*-GAL4 driver, which drives expression in serotonergic neurons, results in formation of aberrant swellings (spheroids) along serotonin-IR neurites (arrowheads). From left to right, columns are dorsal neuropil at most caudal portion of L3F VNC (A3-A7), dorsal neuropil of 10 day adult abdominal VNC, OL of 10day adult, magnification of approximate boxed area in third column images. Scale bar in first, second and fourth columns = 10 μ m, scale bar in third column = 20 μ m. (B) Schematic of dopaminergic and serotonergic cell body location in fly larval abdominal ventral nerve cord. Open circles indicate 5-HT cell bodies while filled circles represent dopaminergic cell bodies. Boxed region represents approximate regions pictured in (C) and (D). (C) 5-HT immunoreactivity in larval VNC driving UAS-Trh in both the serotonergic and dopaminergic neurons using Trh-GAL4 and th-GAL4, respectively. Dorsolateral dopaminergic neurons are indicated with arrows. Ventromedial dopaminergic neurons are indicated with arrowheads. Asterisks distinguish spheroids from cell bodies. (D) 5-HT immunoreactivity in larval VNC driving UAS-Trh in only the dopaminergic neurons using th-GAL4. Dopaminergic cell bodies are indicated as in (C). Note comparable 5-HT immunoreactivity between dopaminergic and serotonergic cells and lack of 5-HT immunoreactive spheroids in the neuropil. Scale bar in (D) is approximately 10 μ m and applies to both images. Abbreviations: VNC = ventral nerve cord, OL = optic lobe, L3F = foraging third instar larva.

Figure 2.

Spheroid characterization reveals gene-dose dependence and temporal characteristics of reversible spheroid formation.

(A) Box-whisker plot demonstrating volume distribution of serotonergic varicosities and spheroids in control and Trh over-expressing L3F abdominal CNS. Average volumes $\pm$ s.d. are presented to the right of the boxes. P-values based on Student's t-test comparison to control group. (B) The number of spheroids present in abdominal serotonergic neuropil is dose-dependent upon transgene expression at L3F. *** $p < 0.001$, Kruskal-Wallis ANOVA. (C) Spheroids are first observed in L3F abdominal neuropil 48 hours after egg lay when UAS-Trh is being maximally driven in serotonergic neurons (Trh-GAL4/Trh-GAL4;UAS-Trh/UAS-

Trh). Error bars represent s.d. (D) Larvae of the sensitized genotype (*Trh-GAL4/Trh-GAL4;UAS-Trh/+*, solid line with circles) are compared to larvae containing a temperature sensitive Tubulin-GAL80 (*Trh-GAL4/Trh-GAL4;UAS-Trh/Tub-GAL80^{ts}*, dotted line with squares) to repress Trh over-expression until shifted from 18°C to 30°C. Both groups were held at 18°C for 48 hours after AEL, then shifted to 30°C for 48 hours, then shifted back to 18°C for 48 hours. Asterisks indicate comparisons between age-matched groups from each data set. Crosses indicate comparison of GAL80-ts timepoints to the 96 hour timepoint ([†]p < 0.05, **p < 0.01, ^{†††}, ***p < 0.001, ANOVA). Error bars indicate s.d. B,C,D: n ≥ 8 larval CNS.

Figure 3.

Serotonergic spheroids are atypical structures exhibiting differential protein localization (A) Spheroids in *Trh*-GAL4;UAS-*Trh* third instar larval abdominal neuropil stained with antibodies against 5-HT and Trh. (B) Spheroids in *Trh*-GAL4;UAS-*Trh*/UAS-h-tau abdominal neuropil stained with antibodies against 5-HT and human tau. Magnified view of boxed region for each shown in (A') and (B'). Scale bar in (A) = 10 μ m. Scale bar in (A') = 1 μ m. (C and D) Examples of plotted pixel intensity taken along a line placed across the spheroids similar to that pictured in (A') and (B'), respectively. (E) Protein localization presented as the pixel intensity taken from the core of the spheroid relative to the intensity taken at the periphery. The intensity values obtained from either end of the line were averaged to obtain a single

intensity value for the periphery of each spheroid. All markers are compared to Trh using Student's t-test, two tailed. Representative images of each marker in one spheroid appear above their bar on the graph. Scale bar = 5 μ m. Antibodies against Trh, 5-HT ($p = 0.002$) and dSERT ($p = 0.06$) all localize to the periphery of the spheroid. UAS-syt-GFP ($p = 0.29$) and UAS-mCD8-GFP ($p = 0.09$) also produce GFP immunoreactivity restricted to the periphery of the structure. UAS-GFP ($p = 0.0002$), UAS-GFP-Atg5 ($p = 0.0000002$) and UAS-h-Tau ($p = 0.0006$) are all enriched in the spheroid core based on GFP and tau immunohistochemistry. $n=5$ spheroids for each marker. Error bars indicate s.d.

Figure 4.

Excess serotonin acting in the cytoplasm induces spheroid formation. (A) Genetic and pharmacological manipulations of the sensitized background. Control and experimental groups contain equivalent dosing of GAL4 and UAS constructs (Control 1: *Trh*-GAL4/+;UAS-*Trh*/UAS-mCD8-GFP, Control 2: *Trh*-GAL4/*Trh*-GAL4;UAS-mCD8-GFP/UAS-*Trh*, ***, $p < 10^{-5}$, number larval VNC shown for each point). (B) Manipulations of 5-HT membrane transporters with and without *Tub*-GAL80^{ts} control. White bars represent constitutive expression of *Trh*-GAL4 from the time *Trh* expression normally begins. In late expression groups GAL4 activity was suppressed by *Tub*-GAL80^{ts} until first larval instar, at which time larvae were transferred to 30°C with or without drug to suppress GAL80 and allow GAL4 activity to proceed. Data points within each group are compared to the constitutively expressing group (black bars). (***, $p < 0.0001$, Student's *t*, two-tailed, number larval VNC shown for each point.) Error bars represent s.d.

Figure 5.

Excess serotonin interacts with canonical protein aggregate forming pathways (A) Interactions of the sensitized background with canonical degenerative and aggregate-associated pathways. Control groups are as in Fig. 4. (**, $p < 0.007$, *** $p < 0.0005$, Student's t , two-tailed; number larval VNC shown). Error bars indicate standard deviation from the mean. (B) Representative image demonstrating ubiquitin immunoreactivity in a confocal thin section taken through the approximate center of a spheroid along the Z-axis in *Trh-GAL4;Trh-GAL4/UAS-mCD8-GFP;UAS-Trh* third instar larval CNS. Scale bar = 5 μm.

Figure 6.

Fenfluramine-induced swellings in mouse are similar to serotonergic spheroids in the fly. (A) 5-HT immunoreactivity in saline-injected mouse frontal cortex. Scale bar = 10 μm. (B) 5-HT immunoreactivity in fenfluramine-injected mouse frontal cortex. Large swellings are easily visible along serotonergic axons. (C) Swellings along mouse serotonergic axons end in terminal boutons (arrowhead) and occur *en passant* (arrowhead in (D) along continuous branches (arrows in D). Scale bar in (C) = 10 μm. (E) Box-whisker plot demonstrating distribution of serotonergic varicosity and spheroid volume in saline and fenfluramine-injected mouse frontal cortex.

Table 1

UAS-Trh induced spheroids are specific to serotonergic neurons

GAL4 driver	UAS-construct	5-HT Spheroids	Spheroids in GAL4-expressing cells
<i>Trh</i>-GAL4	UAS-Trh	+	+
5-HT cells	UAS-DDC	–	–
	UAS-TH	–	–
<i>th</i>-GAL4	UAS-Trh	–	–
DA cells	UAS-TH	–	–
	UAS-Trh	–	–
<i>eg</i>-GAL4	UAS-Trh	–	–
5-HT cells early			
<i>RN-2</i>-GAL4	UAS-Trh	–	–
motor neurons			
109(2)80-GAL4	UAS-Trh	–	–
motor neurons			

Representative images following these manipulations are presented in Supplemental Figure 3.