Analysis of Neurotransmitter Tissue Content of *Drosophila melanogaster* in Different Life Stages

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S *Supporting Information*

ABSTRACT: *Drosophila melanogaster* is a widely used model organism for studying neurological diseases with similar neurotransmission to mammals. While both larva and adult *Drosophila* have central nervous systems, not much is known about how neurotransmitter tissue content changes through development. In this study, we quantified tyramine, serotonin, octopamine, and dopamine in larval, pupal, and adult fly brains using capillary electrophoresis coupled to fast-scan cyclic voltammetry. Tyramine and octopamine content varied between life stages, with almost no octopamine being present in the pupa, while tyramine levels in the pupa were very high. Adult females had significantly higher dopamine content than males, but no other neurotransmitters were dependent on sex in the adult. Understanding the tissue content of different life stages will be beneficial for future work comparing the effects of diseases on tissue content throughout development.

KEYWORDS: *Drosophila melanogaster*, development, tissue content, capillary electrophoresis, neurotransmitters
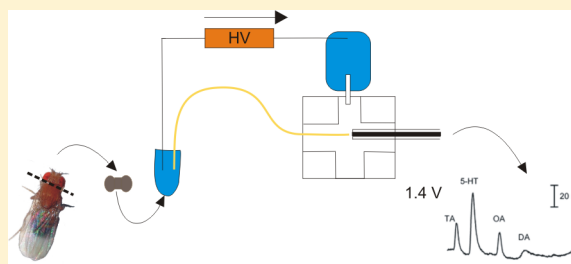
There are four developmental stages for *Drosophila melanogaster*: embryo, larva, pupa, and adult. The larval stage is divided into three instars, during which larva feed and move.[1] During pupation, *Drosophila* attaches to a hard surface and becomes immobile, and larval structures are replaced by adult structures. The pupa then ecloses and emerges as an adult. The central nervous system of *Drosophila* changes during development. In late embryonic development, the primary neurons of the larval brain are developed and the basic structure of the adult brain is laid out.[2,3] As the larvae grow, neurons that will persist into adulthood develop and embryonic stage neurons are pruned.[4] During pupation, larval neuroblasts become adult neurons and there is proliferation and growth of neurons in the brain.[5] Similar to mammals, *Drosophila* have dopamine and serotonin neurotransmission, which is regulated by transporters that are homologous to human transporters.[6,7] *Drosophila* also have tyramine and octopamine, present in humans only as trace amines, that have homologous functions to epinephrine and norepinephrine in mammals.[8] Despite much research into *Drosophila* development, changes in neurotransmitter tissue content over its life cycle have not been characterized.

Neurotransmission has been studied in *Drosophila* by measuring stimulated release of dopamine or serotonin, but tissue content is required to understand how neurotransmitter release relates to the releasable pool of neurotransmitter.[9,10] Separation techniques such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) have been used for tissue content analysis. HPLC requires pooled samples (3−4 heads) because of high mass detection limits.[11,12] With CE, a single head or brain can be analyzed.[13,14] Studying brains is preferred due to the presence of neurotransmitters in the cuticle and the eyes of *Drosophila*. Previous capillary electropohresis studies used amperometric electrochemical detection, which is sensitive but provides little chemical information about the analyte observed. Our lab has coupled CE to fast-scan cyclic voltammetry detection (CE-FSCV), which produces characteristic cyclic voltammograms that aid in analyte identification.[15,16] Previous studies focused on detection of serotonin, octopamine, and dopamine in larval *Drosophila*, but no comparisons have been made to other life stages.[16]

The goal of this study was to compare neurotransmitter tissue content in larval, pupal, and adult wild type (Canton S) brains using CE-FSCV. Tyramine, serotonin, octopamine, and dopamine were quantified and the limits of detection in the 5−16 pg range were sufficient for analysis of single brains. Tyramine and octopamine varied significantly throughout the development of *Drosophila*; in the pupa, tyramine content was high while octopamine content was undetectable. The amount of serotonin or dopamine did not significantly change at any life stage. Adult females had significantly more dopamine than males. Thus, neurotransmitter content can vary by life stage and sex.

■ RESULTS AND DISCUSSION

Capillary Electrophoresis and Fast Scan Cyclic Voltammetry. Capillary electrophoresis with fast scan voltammetry (CE-FSCV) separates analytes quickly and allows small samples to be detected with identification provided by the cyclic voltammograms. The electrode was scanned from −0.4 to 1.4 V and back at 400 V/s every 100 ms for high sensitivity detection of tyramine and octopamine. In order to maximize sensitivity, field amplified sample injection was used by having a high conductivity separation buffer and a low conductivity sample matrix.[17,18] Analytes in this low conductivity solution experience high electric fields and migrate rapidly toward the inlet of the capillary which preconcentrates analytes and decreases peak widths. For the sample matrix, we used deionized water with perchloric acid, which prevents degradation of monoamine neurotransmitters.[19]

Separations of Tyramine, Serotonin, Octopamine, and Dopamine. Figure 1 shows the separation and detection
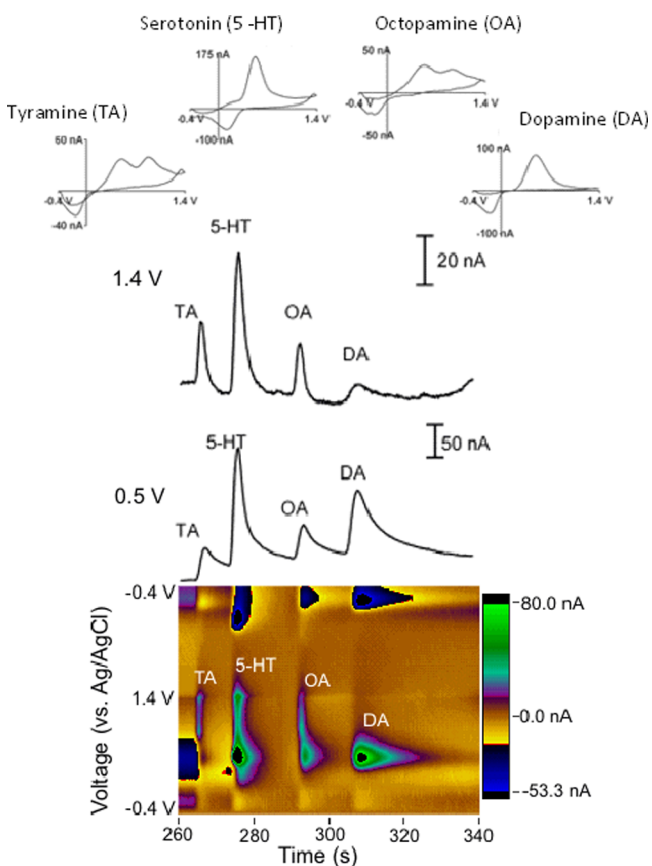

Figure 1. Cyclic voltammograms (CVs), electropherograms, and false color plots for separation of a 100 nM standard mix. Electropherograms at two different potentials are shown so that all oxidation potential peaks can be seen. Neurotransmitters are labeled on the color plot: tyramine (TA), serotonin (5-HT), octopamine (OA), and dopamine (DA).

of 100 nM of tyramine, serotonin, octopamine, and dopamine in 10 μL of 0.5 mM perchloric acid, which takes under 6 min. At the top of Figure 1, the characteristic cyclic voltammogram for each analyte is shown in migration order: tyramine (TA), serotonin (5-HT), octopamine (OA), and dopamine (DA). Tyramine and octopamine each have two peaks, with the primary oxidation peak at 1.3 V and a secondary peak around

0.5 V. Both are well separated which avoids confusion of analyte identity. Serotonin has a single, sharp peak at 0.5 V. Dopamine has a wider oxidation peak around 0.6 V and a reduction peak at −0.2 V. Below the cyclic voltammograms are two electropherogram traces at different voltages: 1.4 and 0.5 V. At 1.4 V, tyramine, serotonin, and octopamine are easily visible, and at 0.5 V all analytes are visible. The peaks for tyramine and octopamine at 0.5 V are secondary peaks, and thus, they last longer at the electrode. For quantitation, primary peaks from the cyclic voltammograms are used.

The current versus time electropherogram (Figure 1) traces differ from those obtained using amperometry, as with amperometry all analytes oxidizing at or below the applied potential would be present. For cyclic voltammetry, only compounds with a current at that given potential are present; thus, not every compound is seen on every trace. In order to view all potentials at the same time, a false color plot is used (Figure 1). The color plot at the bottom of Figure 1 plots applied potential on the y-axis, time on the x axis, and current in false color. The green areas show positive current (oxidation), and the blue areas show negative current (reduction). In the color plots from real samples (Figure 2), other areas of color appear due to unknown compounds present in the fly. The color plot helps identify the analytes of interest and discriminate them from interferents.

Using 100 nM standards, limits of detection were calculated for the analytes. For tissue content studies, the results are often reported in absolute amount of neurotransmitter and the LODs in pg were 16 ± 2 for tyramine, 5.3 ± 0.9 for serotonin, 12 ± 3 for octopamine, and 5 ± 2 for dopamine ($n = 3$). These limits of detection are comparable to previously published work using our CE-FSCV system.[16]

Separations of Neurotransmitters in Different Life Stages of *Drosophila*. Figure 2 shows separations of neurotransmitters in a third instar wandering larva, late pupa, and 72 h old adult. In the larva (Figure 2A), tyramine, serotonin, octopamine, and dopamine were all present in low amounts. The amount of neurotransmitter in Figure 2A was 18.2 pg of tyramine, 12.9 pg of serotonin, 16.7 pg of octopamine, and 16.7 pg of dopamine. In the pupa (Figure 2B), only three peaks were present: tyramine, serotonin, and dopamine. There were no peaks for octopamine where it should migrate. The amount were higher in the pupa than in the larva, especially for tyramine (451 pg) and dopamine (103 pg). In the adult (Figure 2C), all four neurotransmitters were present. The amount of neurotransmitter in this adult was 56.8 pg of tyramine, 22.5 pg of serotonin, 41.0 pg of octopamine, and 69.2 pg of dopamine. While migration times slightly varied due to different capillaries or differences in EOF, migration order is the same for all separations.

Our tissue content values (Table 1) are similar to those previously reported. However, no previous reports of any pupal values are available. Tissue content of dopamine in larva (41 pg/brain) was higher than previously reported (19 ± 2 pg/brain).[16] In adult brains, tissue content (85 pg/brain) agreed with some previously published values (80.7 pg/brain)[20] but not others (10 ± 2 pg/brain).[21] Differences in values here may be due to sample preparation or the inclusion of small parts of the cuticle in our samples. Whole head content was much higher than our value (398 ± 137 pg/brain),[14] which is due to the inclusion of cuticle in the sample.[22]

Serotonin tissue content values for larvae (18 pg/brain) were similar to those in our previous work (23 ± 2 pg/brain)[16] and
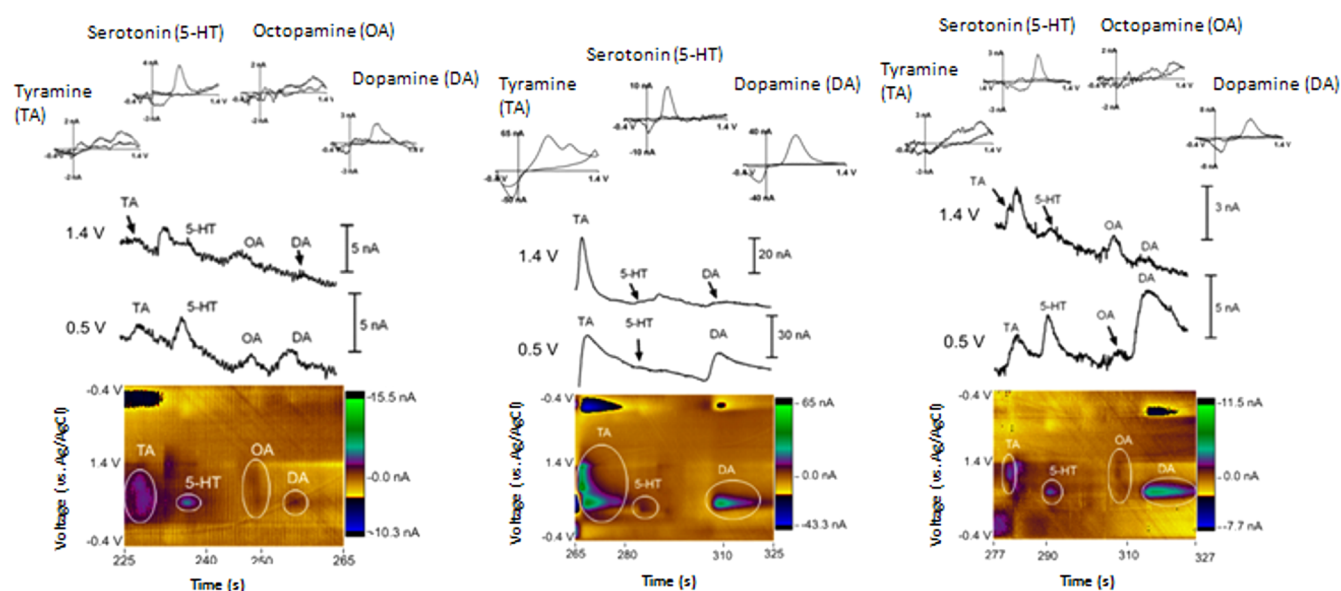
Figure 2. Cyclic voltammograms (CVs), electropherograms, and false color plots for separations of (A) third instar larva central nervous system, (B) late stage pupa brain, and (C) 72 h adult male brain. Neurotransmitters are labeled on the color plot: tyramine (TA), serotonin (5-HT), octopamine (OA), and dopamine (DA).

Table 1. Average Tissue Content Values in Larva, Pupa, and Adult

life stage	tyramine (TA)	serotonin (5-HT)	octopamine (OA)	dopamine (DA)
larva (n = 8)	66 ± 18 pg	18 ± 3 pg	58 ± 19 pg	41 ± 15 pg
pupa (n = 7 −8)	272 ± 57 pg	21 ± 3 pg	not detected	137 ± 57 pg
adult (n = 10)	67 ± 8 pg	22 ± 5 pg	45 ± 7 pg	85 ± 13 pg

those previously reported elsewhere (13 pg/brain).[23] In adult brain samples, there was good agreement between our serotonin values (22 pg/brain) and previously published work (25 pg/brain).[24] Serotonin content in brains was lower than those previously reported in whole head samples (1867 ± 317 pg/head).[14]

Our tyramine value for adults was higher (67 pg/brain) than a previously reported value (7.6 pg/brain),[20] but was lower than values for tyramine from whole heads (109 ± 69 pg).[14] No previous average values for tyramine have been reported in larvae. Differences in tissue content may be due to the age of flies collected. In the same paper with the low tyramine value in adults, a high value for octopamine (263 pg/brain) was reported.[20] Since tyramine is the synthetic precursor to octopamine, high synthesis of octopamine could lead to smaller amounts of tyramine. Octopamine tissue content in larva (58 pg/brain) was not significantly different from our previously published work (38 ± 3 pg/brain).[16] Other laboratories have reported higher values for adults (263 pg/brain)[20] than our whole brain values (45 pg/brain), but Hirsh and Hardie[25] have shown evidence of an unidentified analyte that coelutes with octopamine, causing overestimation of octopamine tissue content. By using FSCV, we were certain of our octopamine peak identification. Our octopamine values were also lower than those of whole head samples (260 ± 61 pg/head),[14] but this could be due to the inclusion of Type II terminals in head samples, which are octopaminergic terminals to muscles that occur outside the brain.

Comparisons of Neurotransmitters in Different Life Stages of *Drosophila*. Figure 3 shows the average amount of tyramine (A), serotonin (B), octopamine (C), and dopamine (D) across the three life stages studied. The average values from each life stage were compared using one-way ANOVA with Bonferonni post-test. Dopamine ($F_{(2, 22)}$ = 2.373, p = 0.1166) and serotonin ($F_{(2, 23)}$ = 0.2022, p = 0.8184) tissue content do not significantly change over the course of development. Tyramine significantly varied by development stage ($F_{(2, 23)}$ = 13.01, p = 0.0002), and content was significantly lower than in the pupae than in either the larva (p = 0.0007) or the adult (p = 0.0004). Octopamine also varied by life stage ($F_{(2, 23)}$ = 7.173, p = 0.0038), with the pupa having significantly less octopamine than either the larva (p = 0.0046) or the adult (p = 0.0232).

The largest differences over development are observed in tyramine and octopamine, where pupae have large amounts of tyramine but almost no octopamine. Octopamine is responsible for locomotion, the fight or flight response, aggression, and feeding in *Drosophila* and other arthropods.[8,26] Tyramine may inhibit locomotion and other octopamine mediated behaviors, especially in larvae.[27] During pupation, there is no movement or feeding; thus, many of the behaviors modulated by octopamine do not occur. In addition, high levels of tyramine might serve to inhibit movement in the pupa.

Tyramine synthesis is controlled by tyrosine decarboxylase (Dmel\Tdc2), which is expressed at low levels throughout development.[28] However, the enzymatic activity of Tdc2 is not known and may be high despite low levels of expression. Tyramine is the synthetic precursor to octopamine; therefore, if octopamine is not synthesized, more tyramine may be present. However, the large difference in tyramine concentration is likely not due solely to reduced synthesis of octopamine, as the amount of tyramine in pupa is greater than the combined
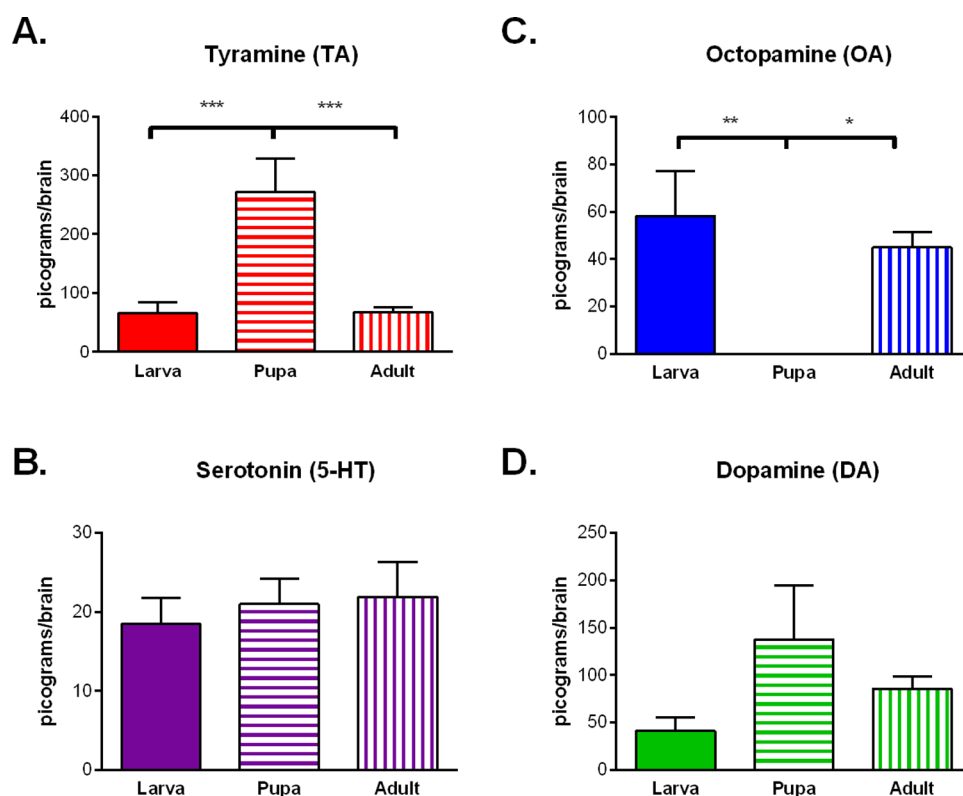
Figure 3. Comparisons of average tissue content across the three tested life stages, larva ($n = 8$), pupa ($n = 7-8$), adult ($n = 10$): (A) tyramine, (B) serotonin, (C) octopamine, and (D) dopamine. One-way ANOVA with Bonferroni's post-test was used to compare the tissue content of each life stage. In panel (A) (F(2, 23) = 13.01, $p = 0.0002$), there is significantly more tyramine in the pupa than in the larva ($p = 0.0007$) and the adult ($p = 0.0004$). In panel (B) (F(2, 23) = 0.2022, $p = 0.8184$), no difference between any life stages was observed. In panel (C) (F (2, 23) = 7.173, $p = 0.0038$), both the larva ($p = 0.0046$) and the adult ($p = 0.0232$) had significantly more octopamine than that in the pupa. In panel (D) (F(2, 22) = 2.373, $p = 0.1166$), no significant difference was observed.
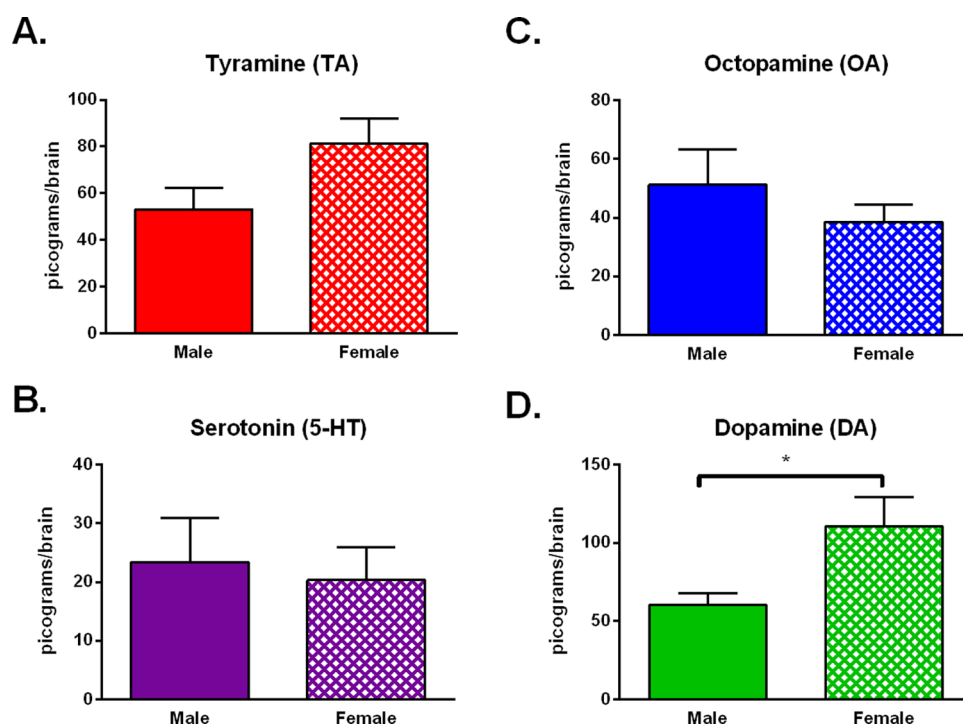

Figure 4. Comparisons of tissue content in male ($n = 5$) and female ($n = 5$) adult Canton S brains for (A) tyramine, (B) serotonin, (C) octopamine, and (D) dopamine. Unpaired t tests were used to determine if the average content of a given neurotransmitter was significantly different in male or female. For tyramine ($p = 0.0793$), octopamine ($p = 0.3671$), and serotonin ($p = 0.7566$), there was no significant difference in tissue content between the two sexes. For dopamine, females had significantly more dopamine than males ($p = 0.0383$).

amount of octopamine and tyramine in the larva. Octopamine is synthesized from tyramine via the enzyme tyramine-β-hydroxylase (Dmel\Tbh). Larva and adults have similar gene expression for Tbh, and pupa at stage 8 (P8) have moderate expression.[28] However, we measured content at P10, where expression is not known and the enzyme activity might not follow the gene expression. Thus, the observed differences in tissue content are not easily predicted by changes in synthesis enzyme gene expression.

Receptor expression can also vary by developmental stage. Increases in the expression of tyramine receptors (Amtyr1, similar to Dmel\TyrR) in the pupal stage have been observed in *Apis mellifera* (the honey bee).[29] The expression patterns of TyrR in *Drosophila* closely follow those of *Apis mellifera*.[29] While receptor expression does not necessarily mean increased content, it may be a good indicator of increased effects of tyramine in pupae. *Drosophila* has several β-adrenergic receptors for octopamine (Octβr) that have different expression patterns throughout the central nervous system. For all three of these receptors (Octβ1R, Octβ2R, and Octβ3R), gene expression in the P10 stage was barely detectable and was significantly higher in the adult.[30] Furthermore, the expression of Octβ2R was much higher than that in the third instar larva than any pupal stage.[30] Thus, the levels of receptor expression for Octβ2R match with the tissue content studies, suggesting little octopaminergic signaling occurs in pupae.

Comparisons of Neurotransmitters in Male and Female Adult Drosophila. Figure 4 shows comparisons of tyramine, serotonin, octopamine, and dopamine tissue content in male and female adult Canton S flies. Only dopamine differs significantly, with more being in females than males ($p = 0.0383$, unpaired t test). Higher dopamine content in females has also previously been observed in rats[31] and *Manduca sexta* (tobacco hornworm).[32] In *Drosophila*, increased dopamine in males alters courtship behavior.[33] Dopamine depletion in females is linked to reduced sexual receptivity,[34] and dopamine is required for female flies to produce pheromones.[35] Therefore, higher dopamine levels in female Canton S flies are likely linked to reproduction.

Tissue content provides a snapshot of neurotransmitter availability and relates to synthesis and tissue stores in the brain. In humans and rats,[36,37] sex related differences are related to neuroanatomy, kinetics of clearance and reuptake, and metabolism of neurotransmitters.[38] Future work with metabolic and transporter *Drosophila* mutants to determine the impact of these mutations on neurotransmitter tissue content is important. In addition, behavioral phenotypes in mutants, such as sex-atypical courting[33,39] and aggressive behaviors,[27,40−42] could signify differences in neurotransmitter content. These mutants could be examined for sex differences and combined with studies of in situ monitoring of reuptake and release kinetics. Understanding the tissue content of wild-type flies can facilitate studies of age related changes in neurotransmitter content or disease-mediated changes in content, such as in Parkinson's disease.

CONCLUSIONS

Tissue content analysis of single larva, pupa, and adult brains by CE-FSCV provides sensitive and accurate determination tyramine, serotonin, octopamine, and dopamine. Furthermore, identification of these neurotransmitters via characteristic cyclic voltammograms provides confidence in peak assignments. Tyramine and octopamine vary significantly over the develop-

ment of *Drosophila*, with high tyramine levels but low octopamine levels in pupae. In adult *Drosophila*, dopamine tissue content is higher in females than males. In the future, CE-FSCV could be used to understand the long-term effects of aging or disease, such as Parkinson's disease, on tissue content.

METHODS

Chemicals. Tyramine, octopamine, serotonin, dopamine, and perchloric acid were purchased from Sigma (St. Louis, MO). Stock solutions (10 mM) of neurotransmitters were prepared in 0.1 M perchloric acid. The separation buffer was 200 mM NaH$_2$PO$_4$ (pH 3.0). The detection cell buffer was 100 mM NaH$_2$PO$_4$ (pH 7.45). Buffer solutions for CE were filtered with a 0.2 μm nylon filter (Alltech, Deerfield, IL).

Fly Homogenate Preparation. Phosphate buffered saline solution (pH 7.4), with trehalose and glucose, was used for dissections. Canton S fly stocks were supplied by the *Drosophila* Stock Center (Bloomington, IN). For larvae, the entire central nervous system (CNS) of a 5 day old, third instar *Drosophila* larva was removed and placed in a Petri dish containing 4 mL of dissection buffer. For pupa, the pupal case was opened. The head was then removed from the body, and any cuticle and visible glial sheath was removed from the brain. For adults, the head was removed from the body, and trachea, cuticle, eyes, and any visible glial sheath was removed.

Sample vials were made by rotating the trimmed end of a gel-loading pipet tip (Eppendorf 20 μL Microloader tips) in a flame to seal the end. A new vial was used for each sample. The CNS was transferred from the Petri dish via pipet, with minimal dissection buffer, to a sample vial containing 10 μL of 0.5 mM perchloric acid. The sample vial was centrifuged at 9000 rpm for 2 min at room temperature. A silver wire was used as a pestle to break up the CNS. The sample vial was then sonicated in a bath sonicator (Fisher Scientific FS30) for 15 min and then inverted over a centrifugal filter (Ultrafree Centrifugal Filters, 0.22 μm, Millipore, Billerica, MA). The sample was centrifuged at 13 200 rpm for 2 min, the sample vial was removed, and the sample centrifuged again for another 2 min. The filtrate was transferred to a 500 μL microcentrifuge tube, and electrokinetic injections were made by placing the end of the separation capillary into this tube. Fly samples and standard samples made in the water and perchloric acid sample matrix were stored on ice in the dark until use to minimize reduce sample degradation.

Instrumentation and Data Analysis. The CE with end column FSCV was built in house and was performed as previously described (see the Supporting Information).[16]

Fast-scan cyclic voltammetry was performed as previously described.[16] Quantitation of analytes in fly samples was performed via post calibration using a 10 μL (0.5 mM perchloric acid) solution with 100 nM of each standard mixture of dopamine, tyramine, octopamine, and serotonin. The response (in nA) of the electrode to the known concentration of the standard mix was used to determine the concentration of analytes in the fly sample. All CVs were filtered at 20 kHz to remove noise.

Statistics. Error bars are shown as standard error of the mean (SEM). Statistics were performed in GraphPad Prism 6 (La Jolla, CA). Unpaired t tests were used to compare means from two groups. One-way ANOVA with Bonferroni post-tests were used to compare three or more groups. Outlier testing was performed on all data sets using ROUT ($Q = 0.1\%$), and definitive outliers were removed.

ASSOCIATED CONTENT

Ⓢ Supporting Information

Methods detailing the complete capillary electrophoresis setup and the modified fly dissection buffer used in this work. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

M.E.D.: sample preparation, performed capillary electrophoresis, and data analysis. E.P.: fly dissections. B.J.V.: supervised experiments and helped prepare this manuscript.

Notes

The authors declare no competing financial interest.

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