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Drosophila as a model system for neurotransmitter measurements

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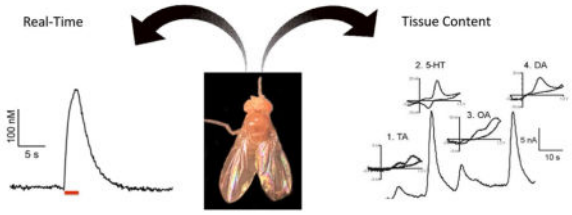
Abstract

Drosophila melanogaster, the fruit fly, is an important, simple model organism for studying the effects of genetic mutations on neuronal activity and behavior. Biologists use *Drosophila* for neuroscience studies because of its genetic tractability, complex behaviors, well-known and simple neuroanatomy, and many orthologs to human genes. Neurochemical measurements in *Drosophila* are challenging due to the small size of the central nervous system. Recently, methods have been developed to measure real-time neurotransmitter release and clearance in both larvae and adults using electrochemistry. These studies have characterized dopamine, serotonin, and octopamine release in both wild type and genetic mutant flies. Tissue content measurements are also important, and separations are predominantly used. Capillary electrophoresis, with either electrochemical, laser-induced fluorescence, or mass spectrometry detection, facilitates tissue content measurements from single, isolated *Drosophila* brains or small samples of hemolymph. Neurochemical studies in *Drosophila* have revealed that flies have functioning transporters and autoreceptors, that their metabolism is different than in mammals, and that flies have regional, life stage, and sex differences in neurotransmission. Future studies will develop smaller electrodes, expand optical imaging techniques, explore physiological stimulations, and use advanced genetics to target single neuron release or study neurochemical changes in models of human diseases.

Graphical Abstract



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Keywords

Drosophila; Dopamine; Octopamine; Serotonin; Optogenetics; Glutamate; Voltammetry; Capillary Electrophoresis

1. Introduction

The complex neural circuitry and vast number of cells within the mammalian brain pose serious challenges to our understanding of behavior, brain development, and neurological disease. Simpler model systems, such as *Drosophila*, *C. elegans*, or zebrafish provide tractable research models for understanding neurological processes, and *Drosophila* in particular are especially attractive because they are easy to genetically alter and are capable of generating complex behaviors.[1] Similar to zebrafish and *C. elegans*, *Drosophila* has a short life cycle and large brood size; however, zebrafish have comparatively few mutants available whereas *Drosophila* and *C.elegans* have large libraries of transgenics/mutants available to researchers.[2, 3] Additionally, *Drosophila,* unlike *C. elegans,* have a central nervous system which makes the fly model advantageous to study comparable behaviors found in mammals, like aggression, sensory processing, and arousal.[4] Flies use the regular canon of neurotransmitters (dopamine, GABA, glutamate, acetylcholine, serotonin, etc.) to communicate between neurons, though flies do possess their own norepinephrine equivalent, octopamine. Furthermore, many pathological features found in human neurodegenerative diseases, like Parkinson's and Alzheimer's, can be found in *Drosophila* disease models.[5, 6] Given that over half of *Drosophila* coding sequences have homologs in the human genome, and that approximately 75% of human disease genes have a counterpart in the fly genome, neurological studies in flies should yield insights into the workings and diseases of the human brain.[7, 8]

1.1 Organization of the *Drosophila* nervous system

The ability to connect neuronal activity to behavior requires a model organism that develops quickly, is genetically pliable, and has a simple neuronal network. *Drosophila* matures in approximately ten days, moving through embryo, larval, and pupal stages to become a fertile adult (Fig. 1A).[9] The larval central nervous system contains 2,000 neurons, as compared to the 100,000 found in the adult stage, and is organized into the ventral nerve cord (VNC) and the protocerebrum (Fig. 1).[10] Three-dimensional reconstructions of the much larger adult brain have revealed 41 anatomical regions and 58 interregional tracts.[11] Like mammalian systems, the *Drosophila* brain utilizes neurotransmitters to communicate between neurons, and neurotransmitters vary by region. In *Drosophila*, dopamine is essential for male courtship behavior, controlling movement, sleep, and learning.[12–15] Immunohistochemistry has detected about 282 dopamine neurons in the adult brain, located in 18 scattered clusters.[16] Octopamine, a derivative of tyrosine like dopamine, is likewise restricted to a limited number of neurons (~150) in the adult brain and regulates aggression and reward in the olfactory system.[17–19] Ten clusters of serotonin neurons exist to mediate behaviors like place memory and aggression.[20, 21] Though dopamine, octopamine, and serotonin are only found in a few hundred neurons, each exerts a profound impact on fly behavior.

1.2 Genetic tools in *Drosophila*

Many genetic tools have been developed that allow researchers to explore the genes and neurons necessary for a functioning brain. The GAL4/UAS (upstream activating sequence) system has proven foundational for targeting gene expression.[22] The GAL4 transgene encodes a transcriptional activator capable of binding to the upstream activating DNA sequence and driving expression of any downstream gene. Expression of GAL4 is dependent on cell-specific enhancer elements; thus, any gene introduced downstream of the UAS element is activated only in the same cells defined by the GAL4 enhancer (Fig. 2A). Because the GAL4/UAS method has great versatility, large collections of stocks have been created that drive GAL4 in different tissues and developmental stages.[23–25] Reporter genes, like green fluorescent protein (GFP), have been used to map anatomical structures and neural networks. To understand specific gene function, libraries of fly strains have been created that use RNAi to target and knockdown any gene of interest.[26, 27]

Use of a simple GAL4 driver line with GFP reporter often labels too many neurons, and additional genetic modifiers are required to refine GAL4 activity. The GAL80 protein antagonizes GAL4 transcriptional activity, and its addition in *Drosophila* restricts GAL4 to fewer sets of cells (Fig. 2B). However, further transcriptional elements must be employed to limit transgene expression to a narrower subset of cells. In the split-GAL4 system, the DNA-binding domain and the activation domain of the GAL4 transcriptional activator are each fused to a leucine zipper motif and expressed independently by two different ‘hemi’ drivers (Fig. 2C).[28, 29] Functionality of the GAL4 protein is restored only in cells expressing both protein domains, which occurs in the overlapping expression pattern of the two ‘hemi’ drivers. The use of the split-GAL4 system has allowed the mapping of restricted neuronal subsets important in behaviors such as sleep and visual memory. [30, 31]

1.3 Developing *Drosophila* as a tool for understanding neurochemistry

The imaging and genetic tools developed so far in *Drosophila* have been able to identify neurotransmitters, neuronal clusters, and genes important for brain activity and disease pathology. However, real challenges exist in measuring neurotransmitter in *Drosophila* as the brain is roughly 54 μm^3 in size, or about 8 nL.[32] This review highlights the neurochemical techniques that have been developed for real-time neurotransmitter measurements and tissue content studies in *Drosophila* and highlights the biological impacts of these neurochemical measurements. Finally, we offer a perspective on future challenges in the field.

2. Real-Time Neurochemical Measurements

Electrochemical methods, such as constant-potential amperometry, chronoamperometry, and fast-scan cyclic voltammetry (FSCV), are popular tools to monitor real-time changes of neurotransmitters in the brain and can be performed with sub-second temporal resolution. FSCV scans through voltages, provides 100 ms time resolution, and a cyclic voltammogram that identifies the analyte, while amperometry is less selective, applying a constant voltage, but provides high temporal resolution and is best for measuring quantal size. Chronoamperometry steps to different voltages, so it provides a ratio of oxidation/reduction peak to aid in analyte detection, but is not as selective as FSCV. Carbon-fiber

microelectrodes (CFMEs) are commonly used for measuring neurochemicals because of their biocompatibility, sensitivity, favorable electrochemical properties, and small size, typically less than 10 Fm.[33] CFMEs measure from the extracellular space, so they are best for measuring volume transmission, and the background subtraction of many techniques makes it more appropriate for measuring fast neurotransmitters including dopamine, serotonine, histamine, and norephinephrine, as well as rapid neuromodulators, like hydrogen peroxide and adenosine.[34–38] Because there are no stereotaxic coordinates or brain atlas for flies, initial experiments are often performed in flies that that express GFP in the desired region, which provides a target for electrode implantation.

2.1 Electrochemical measurements of uptake

The first real-time neurochemical measurements in *Drosophila* were performed by the Ewing group, which measured uptake of exogenously applied dopamine *in vivo* in adult flies.[39] Dopamine was applied to the protocerebral anterior medial (PAM) region and clearance time by the dopamine transporter (DAT) determined (Fig. 3). Peak concentration after cocaine application, a DAT inhibitor, was significantly larger in *fumin,* a DAT knockout, than wild type demonstrating large doses of cocaine are an effective blocker of DAT. The Venton group also studied dopamine clearance in *Drosophila* larval ventral nerve cords (VNC) *ex vivo.*[40] The entire central nervous system (CNS) of the larva was removed, a CFME implanted from one end, and a picrosptritzing pipette containing dopamine from the other end. While uptake measurements can be made in any fly without genetic modifications, reproducibility is challenging because exact placement of the pipette with respect to the electrode is difficult.

2.2 Optogenetically Stimulated Neurotransmitter Release

In rodents, electrical stimulation is commonly used to activate select neurons by applying a stimulation at the cell bodies and measuring at the terminals. While *Drosophila* neurons can be activated by electrical stimulation, their brain is too small to stimulate specific neurons. Thus, activation of specific cells in *Drosophila* is typically performed with optogenetic, a combination of optical and genetic tools, that uses a light sensitive protein to activate neurons in genetically defined cells with millisecond precision.[41] Light stimulation opens ion channels, allowing cations such as sodium and calcium to enter the cell, depolarize the membrane, and cause neuronal firing and exocytosis.[42] In mammals, channels are typically expressed using a viral vector, such as an adeno-associated virus, to deliver the transgene into target cells.[43] However, in *Drosophila*, the GAL4/UAS system is used to make stable lines that express the channels in specific neurons.

The Venton group pioneered using optogenetic stimulation and FSCV to measure real-time changes in monoamines in the *Drosophila* larval VNC.[44] First, Channelrhodopsin-2 (ChR2), a blue light sensitive channel, was expressed specifically in serotonin neurons containing the synthesis enzyme tryptophan hydroxylase (Tph). Regulation of the serotonin system in *Drosophila* was analogous to mammals; short term release was maintained primarily by uptake while long term release was maintained by synthesis.[45] Similarly, ChR2-mediated stimulation of dopamine was measured in larval dopamine cells expressing tyrosine hydroxylase (TH).[46] Dopamine release was decreased by reserpine, which blocks loading of

neurotransmitters into vesicles, proving it was vesicular. Optogenetic stimulation also caused pH shifts, similar to mammalian systems where pH shifts are observed after electrical stimulation. [47]

The Ewing group developed ChR2 optical stimulation to amperometrically monitor octopamine release in the *Drosophila* larval neuromuscular junction (NMJ).[48] The NMJ was carefully dissected using the fillet preparation and individual octopamine terminals visualized with mCherry (promoter: *Tdc2*-GAL4), a red fluorescent marker. Optogenetic stimulation caused spikes of release, which were analyzed to determine the number of octopamine molecules released per vesicle. Incubation with tyramine, an octopamine precursor, dramatically increased octopamine release. Complementary electrophysiological measurements at the NMJ have likewise highlighted the importance of octopamine in eliciting muscle depolarization. Application of 10 mM octopamine evoked an excitatory junction potential, a response that was diminished in a mutant for an octopamine receptor, *octb2R*, or the octopamine synthesizing enzyme *tbh*.[49]

Blue light stimulation suffers from a number of limitations, including limited penetration depth, strong absorbance by endogenous chromophores,[50] and the photoelectric effect, which causes an error at the electrode during FSCV.[51] Moreover, to achieve functional levels of ChR2, a homozygous transgenic line was necessary, with two copies of the channel.[52] To circumvent these problems, a red light activated channel, CsChrimson, was employed (Fig. 4). The lower energy of red light did not produce stimulation artifacts at the electrode,[51] provided better tissue penetration,[53] and required only a single copy of CsChrimson. Different brain regions were studied, including the larval protocerebrum, which develops into the adult protocerebrum that controls olfactory learning and memory, and the VNC, analogous to the vertebrate spinal cord, which contains clusters of motor neurons that are innervated in a highly organized manner.[54] More optogenetically-stimulated dopamine release was detected in the larval VNC than in the protocerebrum, demonstrating strong neurotransmission in the motor neurons.[51] Using CsChrimson, dopamine release was detected with a single stimulation pulse (4 ms) in both the VNC and protocerebrum,[51] whereas at least 10 stimulation pulses were needed with ChR2.[55] Octopamine release and uptake in *Drosophila* larval VNC were also studied,[56] and octopamine release was vesicular and stimulation frequency dependent, similar to dopamine and serotonin release.

2.3 Chemically Stimulated Neurotransmitter Release

An alternative to inserting a light-activated channel is to express a chemically-sensitive channel in specific *Drosophila* neurons. $P2X_2$, a ligand-gated cation channel, is a mammalian ATP receptor that is not encoded in *Drosophila*.[57] $P2X_2$ is activated by ATP, causing membrane depolarization and neuronal firing, but not apoptosis. The amount of dopamine released using ATP/$P2X_2$ stimulation was approximately 400 nM, smaller than with CsChrimson stimulation.[58] Pharmacological manipulation of dopamine synthesis and uptake demonstrated both release and uptake are important for replenishing the dopamine releasable pool.

Another option is to use pharmacological agents to evoke endogenous release without expressing any exogenous channel. For example, nicotine and acetylcholine activate

nicotinic acetylcholine receptors (nAChRs) to evoke neurotransmitter release.[59] Fuenzalida-Uribe *et al.* studied nicotine stimulated octopamine release in adult *Drosophila* brains using high-speed chronoamperometry.[60] As 5 mM nicotine was pressure ejected 1 mm from the brain surface, the efflux of octopamine was recorded in the mid-ventral side of an isolated adult brain. Octopamine was important for nicotine-mediated startle reflex. In the larval VNC, nAChR agonists, such as acetylcholine, nicotine, and neonicotinoids insecticides, stimulated dopamine release.[61] Neonicotinoid-stimulated dopamine release was lowered in Drosophila nAChRs subunits mutants, EMS1, α1 subunit mutant and EMS2, β2 subunit mutant which are known to resistant to neonicotinoids insecticides. The benefit of using nAChR stimulation is that no genetic manipulation is required; thus, any fly can be tested.

2.4 Biological insights gained from real-time measurements of neurotransmitters

While many of the studies of *Drosophila* neurochemistry have developed new techniques, these studies are already providing new insight into the dynamics of neurotransmitter release and the genes that regulate neurotransmission. Early electrochemical studies proved the similarity of *Drosophila* and mammalian system, establishing that monoamine release is due to exocytosis[44] and clearance due to uptake via transporters.[62] These results are critical for establishing *Drosophila* as a good model system for studying neurotransmitter release and uptake. Here, we highlight some of the biological insights gained from real-time neurotransmitter studies.

2.4.1 Psychostimulant and antidepressant activity—Monoamine transporters regulate extracellular levels and are targets for psychostimulants and antidepressants. Pharmacology is slightly different in the fly, where psychostimulants like cocaine are potent inhibitors of the serotonin transporter (SERT, K_i=464 nM) as well as dopamine transporter (DAT, K_i=2660 nM),[63] their main target in mammals. Antidepressants given to humans, like selective serotonin reuptake inhibitors (SSRIs), often target SERT. The uptake kinetics for *Drosophila* DAT were determined using optogenetic stimulation and Michaelis-Menten modeling.[40] The maximum uptake rate (V_{max}) was higher in the protocerebrum than the VNC but the affinity of dopamine (K_m) for DAT was similar in each region.[51] For SERT, V_{max} and K_m in the larval VNC were similar to mammalian values.[44] In adult *fumin* flies, higher dopamine concentrations were detected because they lack functional DAT.[62] Similarly, in *fumin* larvae, clearance rates of dopamine were slower.[40] Methylphenidate, a drug used to treat attention deficit disorder and narcolepsy,[64] inhibits DAT with a similar binding affinity to cocaine but has a longer half-life.[65] Methylphenidate is therefore less addictive and is a proposed treatment for cocaine addiction. The clearance rate of dopamine was significantly slower after oral administration of methylphenidate and methylphenidate-treatment reduced the effects of cocaine.[66] Future studies can examine how genetic mutations in the transporter affect the actions of uptake inhibitors, which could be important for understanding human variations in response to drugs such as SSRIs.

2.4.2 Autoreceptor regulation—Another important finding from real-time neurotransmitter measurements in *Drosophila* was that flies have functioning D2 autoreceptors, similar to mammals.[67] Mammalian D2 dopamine receptors (D2R) are presynaptically expressed and act as autoreceptors, regulating dopamine release. *Drosophila*

D2-like receptors have homologous amino acid sequences to mammals,[68] but their cellular localization and ability to regulate dopamine release was not known. D2Rs are associated with locomotion, learning, and grooming[69] and the D2R agonist bromocriptine improves locomotion in Parkinson flies.[70] Bromocriptine also significantly decreased optogenetically stimulated dopamine in the larval VNC and D2R antagonists, such as flupenthixol and butaclamol, had the opposite effect, increasing release.[67] Thus, D2R functions as an autoreceptor. Autoreceptors are critically important for regulating dopamine and serotonin release and are the targets of many human drugs, including antipsychotics, so *Drosophila* could be a good model system to study how genetic mutations in D2Rs affect the efficacy of drugs.

2.4.3 Octopamine function—Most studies have focused on dopamine or serotonin, as they also signal in mammals. However, octopamine, a homologous neurotransmitter for the vertebrate transmitter norepinephrine, is also important because it modulates neuromuscular transmission and locomotion. Octopamine was released from Type II varicosities in the neuromuscular junction, on average about 22,700 molecules per vesicle.[48] In the larval VNC, octopaminergic neurons were located mostly in the middle of the abdominal section and octopamine release was vesicular and stimulation frequency dependent.[56] The half-life for octopamine was faster than serotonin, which is interesting because no octopamine transporter has been identified and there is no evidence that other transporters, such as SERT, are involved in octopamine clearance. Measurements in adults have also revealed that octopamine is important for a nicotine-induced startle response, which was not observed in flies deficient in octopamine.[60]

3. Tissue Content Measurements

Tissue content studies that quantify multiple neurotransmitters and their metabolites provide another critical piece of information about neurotransmission. Typically, high-performance liquid chromatography with electrochemical detection (HPLC-EC) has been used for separation and quantification of neurotransmitters in *Drosophila* [71] but samples are typically pooled due to high mass detection limits.[71, 72] Capillary electrophoresis (CE) is a popular separation method for neurotransmitters and metabolites, that separates charged molecules according to their charge and size.[73] Neutral molecules can also be separated by adding in a pseudostationary phase, such as sodium dodecyl sulfate (SDS), in a technique called micellar electrokinetic chromatography (MEKC).[74] The advantages of CE are high resolution, low sample volume requirement (nL to fL compared to FL for HPLC), and high sensitivity. [75] In this section, we will focus on capillary electrophoresis studies coupled to electrochemical detection (EC), laser induced fluorescence (LIF), and mass spectrometry (MS) for neurochemical content analysis in *Drosophila*.

3.1 Capillary Electrophoresis – Electrochemical Detection

The Ewing group pioneered using CE coupled with EC detection to identify neurotransmitters in *Drosophila*. They employed MEKC coupled with amperometric electrochemical detection and identified tyramine, serotonin, dopamine, and the dopamine precursor L-DOPA.[76] Further optimization of the run buffer allowed 14 neurochemicals to

be separated, including *N*-acetylated metabolites for the first time.[77] Inactive mutants (*iav*^{1}), defective in locomotor activity, had lower tyramine, octopamine, and *N*-acetyloctopamine content than Canton S wild types, which is biologically important because octopamine controls locomotion and is in the neuromuscular junction.[72]

To ease the burdens of dissection of multiple brains, many studies are performed on whole heads; however cuticle and eye pigments interfered with separations in head homogenates and thus isolating brains is better.[74] The *Drosophila* brain was further dissected into three different brain regions: optic lobes, central brain, and posterior superiormedial protocerebrum (PPM1) region. The amount of dopamine was higher in the PPM1 than in the central brain or optic lobes.[78] Sample preparation was an important consideration for tissue content experiments, as enzymes might break down neurotransmitters after tissue collection. An alternative freeze-drying method facilitated better dissections and preserved neurotransmitters so they would not degrade.[79] In freeze-dried dissected brains, contamination peaks from the eye pigment did not occur (Fig. 5A).

Amperometric detection lacks chemical selectivity and therefore identifying peaks relies on comparing migration times with an internal or external standard. CE with FSCV detection overcomes the problem of identification, as the cyclic voltammogram gives a signature of the analyte (Fig. 5B). Although FSCV is not as sensitive as amperometry, field–amplified sample injection can be applied by diluting the sample with acetonitrile, which enhances loading of sample into the capillary. Thus, *Drosophila* content was determined in a single larval CNS.[80] Levels of octopamine and tyramine differed by life stage (Fig. 5B).[81] Further studies optimized detection of histamine, carcinine, and dopamine from different tissue types including brain, eyes, and cuticle.[82]

3.2 Capillary Electrophoresis – Laser Induced Fluorescence

CE with laser induced fluorescence (CE-LIF) detection has high sensitivity and has been used to analyze microdialysis samples from vertebrates.[83, 84] While LIF is sensitive, most neurochemicals are not fluorescent and thus must be derivatized. The Shippy group used fluorescamine derivitization and CE-LIF to measure amino acid content in *Drosophila* hemolymph, a fluid which is analogous to the blood in vertebrates (Fig. 5C).[85] Hemolymph samples are extremely low volume (50–300 nL in larvae) so a homemade sampling probe was developed to pierce a single larval body cavity with tubing and cover the sample with oil to prevent evaporation.[86] Amino acids were quantified in control Oregon-R flies and *genderblind* (*gb*) mutants, where a cysteine-glutamate transporter mutant causes bisexual behavior. A nanoliter sampling technique was developed to collect 25 nL of hemolymph from a dorsal abdominal incision of adult *Drosophila* (Fig. 5C). Thiols such as glutathione and cysteine were present in the mM range in hemolymph.[87] CE-LIF is a good method for these volume limited samples.

3.3 Capillary Electrophoresis – Mass Spectrometry

CE coupled with mass spectrometry (MS) is powerful because it can detect low concentrations in a volume limited sample, while providing a mass to charge ratio to identify compounds. Electrospray ionization (ESI) interfaces well with the flowing stream of CE,

although care must be taken to ground the CE voltage before the ionization.[88] Some common CE separation buffers, such as TES and borate, as well as SDS surfactants, are non-volatile and cause severe ion suppression with ESI.[89] CE-MS was used to determine the concentration of neurotransmitters, metabolites, and drugs in adult *Drosophila* treated with methylphenidate, commonly called Ritalin.[90] The amount of tyramine and octopamine increased after methylphenidate, but there were no changes in *N-acetyl* metabolites. As CE-MS grows and commercial instruments become more available, more applications will likely be developed for neurotransmitter tissue content measurements.

3.4 Biological insights gained from separations studies

Tissue content studies provide important insight into levels of neurotransmitters and drugs. For example, ratios of different neurotransmitters and metabolites have been determined and N-acetyl metabolites were identified for the first time, confirming monoamine metabolism is different in flies than mammals.[77] Studies of hemolymph measured circulating amino acid levels and revealed that genetic mutations can dramatically alter these levels.[86] Separations are also the only method to quantify how much drug gets to the brain when it is fed orally, and studies have confirmed about 2% of the oral dose reaches the brain.[66] Here, we highlight some of the biological insights gained from these techniques.

3.4.1 Sex, regional, and life stage differences in neurotransmitter content— Tissue content studies have demonstrated that neurotransmitters vary by sex, region, and life stage. Dopamine tissue content was higher in *Drosophila* females,[81] similar to rodent models, where evoked dopamine released was significantly higher in females.[91] In adults, tissue content varied by brain region[78], with 25-fold higher dopamine content in the PPM1 region, rich in dopamine neurons, compared to the central brain. When looking at neurotransmitter differences during development, dopamine and serotonin did not change but pupae had large amounts of tyramine but no octopamine.[81] This finding is consistent with octopamine being important for locomotion and feeding since pupae are immobile and do not eat. Additionally, tyramine is a synthetic precursor to octopamine, thus tyramine levels may build up if octopamine is not being synthesized.

3.4.2 Histamine metabolism—Histamine is a transmitter in *Drosophila*, found in both the eyes and brain, that regulates visual transduction.[92] Histamine is metabolized by β-alanylation, forming carcinine by N-β-alanyl-dopamine synthase (Ebony) which also converts dopamine to a β-alanyl dopamine (BADA). β-alanyl-dopamine hydrolase (Tan) converts carcinine and BADA back to histamine and dopamine. Histamine content was higher in the eyes compared to the brain and cuticle, but there were no significant differences in carcinine content.[82] However, carcinine content was higher in *tan*^{3} mutant flies, especially in the eyes. Tan and Ebony are more highly expressed in the eye than the brain so these differences correspond to gene expression. Overall, these studies provided insight into histamine and dopamine metabolism.

3.4.3 Correlating drug and tissue content—Measuring the drugs that reach a brain in *Drosophila* is quite challenging and CE-MS allows both drug levels and neurotransmitter content to be correlated. The tissue content data validated that different feeding dosages

changes the amount of methyphenidate in fly brains.[90] Methylphenidate increased neurotransmitter levels with dose until the effect saturated after feeding around 20 mM. In addition, a hydroxylated metabolite of methylphenidate was identified, a different metabolism pathway than in mammals.

4. Future Directions for Neurotransmitter Detection in Drosophila

Research on neurochemical monitoring in *Drosophila* started as an analytical challenge, as there were not good existing methods to measure in the small brain of *Drosophila*. Even today, there are still several methodological challenges to be overcome to improve the use of *Drosophila* for studies of neurotransmission. In addition, there are biological challenges and advantages of *Drosophila* that can be harnessed to make it even more of a valuable model organism for neurotransmitter measurements. In this section, we address some of the future challenges and directions of *Drosophila* neurotransmitter research.

4.1 Challenge 1: Measurements in discrete regions

While CFMEs have been the standard electrochemical tool, the size of their tips is large compared to specific *Drosophila* regions. For example, the mushroom bodies of the adult brain have discrete parts including the vertical and medial lobes, calyx, and peduncle. Ideally, the electrode is localized in one discrete area to determine how different clusters of neurons control behaviors. These areas are only about 10 μm wide and the typical 7 μm diameter electrode is likely to destroy or displace much of the tissue. In addition, while octopamine have been measured in the central complex, it too is made up a fan shaped and ellipsoid body, and the ellipsoid body also has a diameter that is on the scale of 10 microns.[60] Therefore, there is a push to develop smaller nanoelectrodes. One strategy is to etch carbon fibers, with a tip diameter less than a micron, that are insulated to form a disk electrode.[93] Electrodes can be electrochemically or flame etched (Fig. 6A),[94, 95] but both methods require much skill to obtain the perfect size tip. Etched electrodes have been used to measure exocytosis in a single synapses in cultured neurons.[95] An alternative strategy is to use carbon nanopipettes, where carbon is grown on the inside of a pipette that is then etched. The Venton lab demonstrated CNPEs, with 200–400 nm tips, for larval VNC dopamine measurements (Fig. 6B–C).[96] The challenge with all nanoelectrodes is that current scales with surface area in electrochemistry; thus, smaller electrodes give smaller signals and electrode development strategies are needed, such as the use of nanoparticles, to increase sensitivity. The ultimate goal is small, sensitive electrode localize in discrete regions or measure from a single neuron.

Genetic tools are also useful to reduce the number of active neurons to probe the response of single neurons or discrete regions. The split-GAL4 system separates the transcriptional GAL4 activator into two genes, both of which need to be expressed by hemi-drivers to drive UAS-gene activity. The split-GAL4 system has been used in genetic screens to pinpoint single neurons important for behaviors such as walking and sleep[30, 97, 98]. Thus, using the split-GAL4 system to drive light-activated channels would facilitate accurate, real-time measurement of neurotransmitter release from specific regions or even single neurons. In *Drosophila*, the exact number and distribution of neurons is conserved, which makes it

possible to manipulate a single neuron reproducibly. With the co-expression of light-activated channels and RNAi-elements, which knockdown genes of interest, studies could tease out how genes regulate neuronal activity, neurotransmitter release, and behavior.

4.2 Challenge 2: *In vivo* measurements of physiologically-stimulated release

The first study of dopamine uptake in flies was actually performed *in vivo*,[39] but all endogenous release experiments have been performed *ex vivo* with the brain removed from the fly. *In vivo*, flies can be prepared in a "fly collar"[39] that immobilizes their heads but allows some behaviors. Several challenges are presented by *in vivo* preparations. First, the entire brain, as well as individual structures in *Drosophila*, are encased in a glial sheath,[96] which is tough and must be removed to implant electrodes. While collagenase can be used to soften the tissue, it might also destroy neuronal connections and so is best avoided. Second, the brain can dry out, and there needs to be an electrical connection to a reference electrode, so a drop of solution is maintained on the head. Third, optogenetic studies might be challenging, as light needs to penetrate into the neurons. However, the red light that activates CsChrimson can penetrate to suitable depths for *in vivo* studies.[99]

Drosophila is a good model system for studying complex behaviors and in particular sensory responses.[100, 101] Several studies have made use of the immobilized flies to measure flight behavior and identify important neural circuitry upon odor or visual stimulations.[101-103] A future direction would be to use these head-fixed *in vivo* experiments to understand how physiological stimuli elicit neurotransmitter release.

4.3 Challenge 3: Expanding the toolbox to other neurotransmitters/neurochemicals

The most used tools for *Drosophila* neurotransmitter measurements are electrochemical measurements and separations, which are useful but have limitations. Direct electrochemistry is limited to electroactive species, or a biosensor can be constructed to turn non-electroactive molecules into electroactive products.[104] For separations, electrochemical detection is similarly limited to electroactive species and fluorescence detection requires molecules that can be easily tagged (typically amines).[75, 83] Mass spectrometry detection better identifies analytes but for electrospray, buffers and salts increase the surface tension of droplets and reduce the volatility, decreasing sensitivity; thus, buffer optimization is critical and more volatile buffers are preferred. Other techniques being developed, such as optical sensors and mass spectrometry imaging, have the potential to detect other analytes and multiple analytes simultaneously.

Optical methods have been recently developed for monitoring neurotransmission. Fluorescent dyes have been synthesized to detect neurotransmitters in the synapse,[105, 106] but they have not yet been applied to *Drosophila*. These dyes, called Neurosensors, have been developed for dopamine and norepinephrine[106], and there is another class of dyes that respond to zinc, glutamate, and the pH shift of exocytosis to monitor only neurotransmitters in the synapse[107]. Another approach is genetically encoded biosensors, that use modified proteins that change fluorescence when neurotransmitter binds.[108] Genetically-encoded biosensors have been made for glutamate, the most abundant excitatory neurotransmitter [109]. These genetically-encoded biosensors provide a unique understanding of the

neurotransmitter available at the synapse, which is complementary to the information about neurotransmitter overflow in the extracellular space that is typically measured with microelectrodes. Synaptic ATP levels have been monitored in real time in *Drosophila* and *C. elegans.* Genetically-encoded biosensors have rapid temporal resolution and high spatial resolution, but are best used in *ex vivo* preparations, which pairs well with whole brain ex vivo *Drosophila* preparations. More genetically encoded sensors are being developed and protein engineering performed to expand the available colors to facilitate multi-analyte measurements[110].

Another possible method to understand more about neurotransmission is mass spectrometry imaging. Mass spectrometry provides the molecular mass of the compounds it detects, and MS/MS techniques are even more powerful to fragment and identify molecular structures. Matrix-assisted laser desorption ionization (MALDI) imaging has been used for *Drosophila*, mostly to identify lipids in the brain[111]. These lipids are important for controlling exocytosis and thus understanding them is crucial to understanding the process of neurotransmission. Secondary ion mass spectrometry (SIMS) imaging has also been used to identify the spatial distribution of lipids, eye pigments, and diacylglycerides. Mass spectrometry provides good spatial resolution, but is typically performed on fixed tissue[112].

4.4 Challenge 4: Studies of disease models related to human health

Many *Drosophila* models have been made of human diseases; *Drosophila* models of Alzheimer's, Parkinson disease, and restless leg syndrome exist that have similar phenotypes to higher order model systems.[5, 6, 113] For the example of Parkinson disease, several genetic mutation models have been made, with mutations in *parkin* or *pink1* genes that mimic mammalian mutants. Recent evidence in *pink1* mutants using dopamine clearance and tissue content measurements, as well as behavioral and genetic studies, suggests there are early changes in DAT expression and dopamine levels during early adulthood in these Parkinson models[114]. Genetic modifications of *Drosophila* are relatively easy, and models can be made with either permanent or inducible gene inactivation.[115] The advantage of *Drosophila* is the well mapped anatomy; for example, if you want to study cell death during a neurodegenerative disease, you can easily track exactly which populations die. By expanding neurotransmitter measurements into these disease models, we will better understand exactly how disease phenotypes affect neurotransmission. Both real-time measurements and tissue content measurements are key to pinpointing how neurotransmission malfunctions during diseases.

6. Conclusions

Drosophila is an important model system for understanding neurotransmitter changes because it has a high homology to mammals, relatively complex behaviors, and a well-defined anatomy. Real-time measurements of neurotransmitters can be performed in *Drosophila* with electrochemistry and separation methods are important for understanding tissue content. Overall, the initial studies of neurotransmission in *Drosophila* have shown it is similar to mammals, regulated by autoreceptors and transporters, and thus it will be an

important model system for understanding monoamine regulation and dysfunction in disease.

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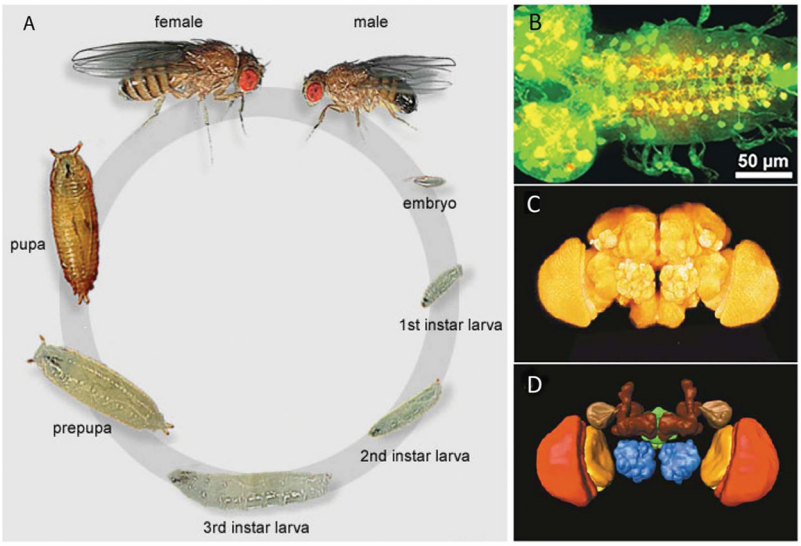

Figure 1.

(A) Drosophila melanogaster develops in approximately 10 days, moving through embryo, three larval, pupal, and adult stages. (B) Ddc-GAL4 drives GFP expression (green) in dopamine and serotonin neurons of the larval brain. Antibody detecting serotonin (red) present; overlap of cells expressing GFP and serotonin appears yellow. (C) Three-dimensional imaging of the adult brain reveals a few distinct anatomical regions. (D) Anatomical regions within the brain were labeled. Dark brown: mushroom body; blue: antennal lobe; green: central complex; red: medulla; orange: lobula. Figures reprinted from ref [6] and [27], with permission of Elsevier.

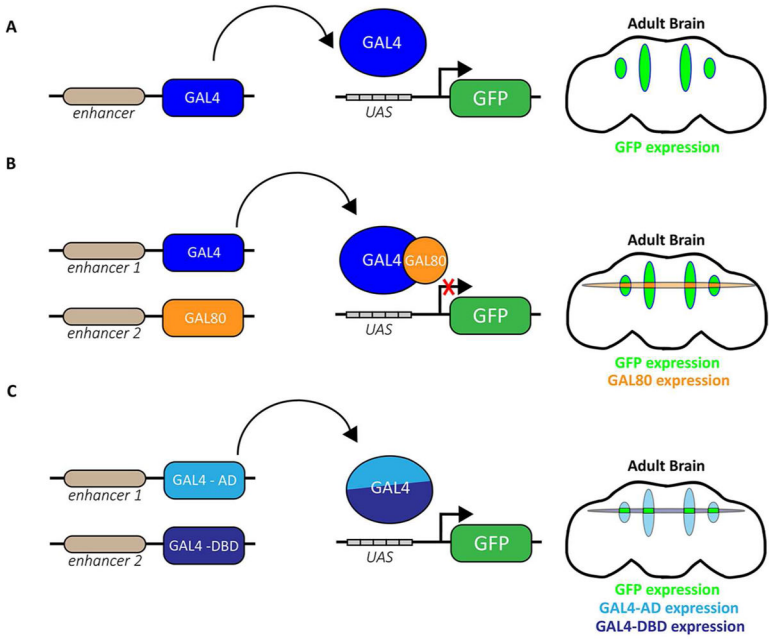

Figure 2.

(A) In flies carrying both the GAL4 (blue) and the UAS constructs, GAL4 will drive expression of the transgene. In this example, GAL4 drives expression of transgenic GFP in four regions of the adult brain. (B) GAL80 (orange) represses GAL4 transcriptional activity, and in cells expressing both GAL4 (blue) and GAL80, transgenic GFP expression will be restricted. (C) With split-GAL4, transgenic GFP (green) will only be expressed in the limited number of cells expressing both the GAL4 activating domain (GAL4-AD, light blue) and the GAL4 DNA-binding domain (GAL4-DBD, dark blue).

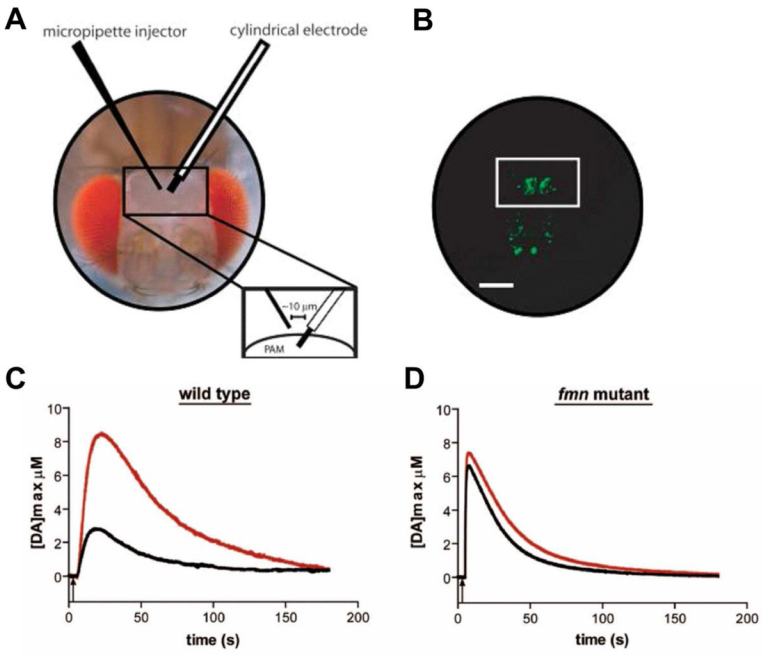

Figure 3.

In vivo measurement of exogenously applied dopamine. (A) Adult fly head after microsurgery exposing PAM neurons (scale bar = 100 μm). (B) Fluorescence image of microsugeried fly head showing GPF expressed in dopaminergic neurons and white box highlights PAM neurons. (C) Representative wild type (WT) data of exogenously applied dopamine trace before (black line) after (red line) the brain was exposed to 1.0 mM cocaine. (D) Exogenously applied dopamine in fumin mutant flies, where dopamine concentration is significantly increased compared to WT. Figures modified from ref [39], with permission of American Chemical Society.

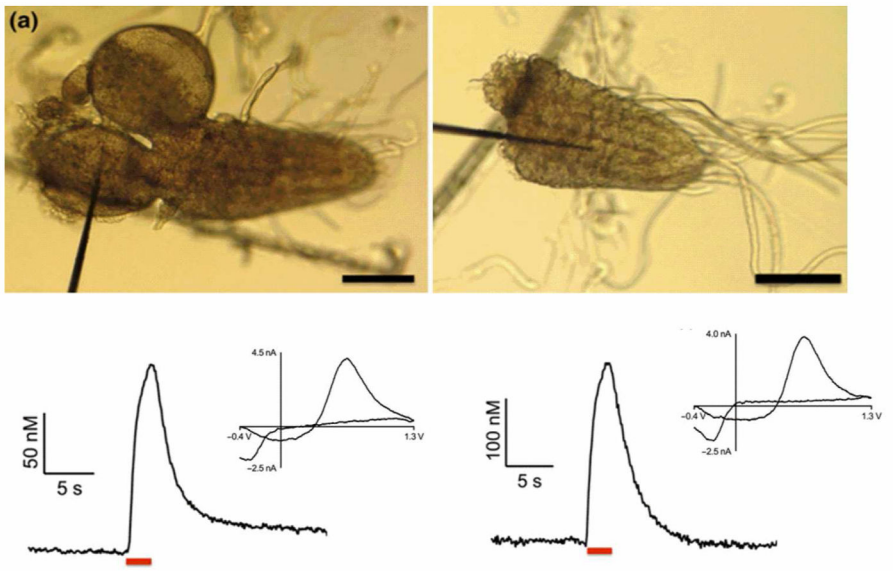

Figure 4.

CsChrimson stimulated dopamine release from Drosophila larvae. (A) CFME in protocerebrum. Scale bar is 50 μm (top). Concentration trace of CsChrimson evoked dopamine release in the larval protocerebrum (2s continuous stimulation) with cyclic voltammogram (CV) of released dopamine (bottom). (B) CFME in larval VNC. Scale bar is 50 μm (top). Concentration trace and CV of CsChrimson evoked dopamine release in the larval VNC (2s continuous stimulation) (bottom). Figures modified from reference [51], with permission of John Wiley and Sons.

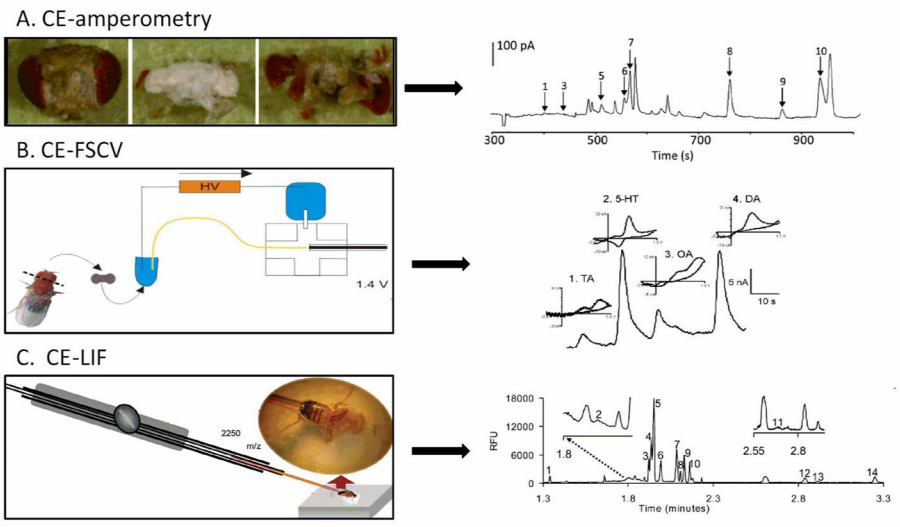

Figure 5.

Neurochemical tissue content using different separations. (A) Image of a freeze-dried adult Drosophila head (left), extracted brain (center), and the cuticle (right). Electropherogram of freeze-dried dissected 15 brain homogenates with 10 Identified peaks (right) (1) dopamine, (2) salsolinol, (3) N-acetyloctopamine, (4) octopamine,(5) N-acetylserotonin, (6) N-acetyltyramine, (7) N-acetyldopamine, (8) L-DOPA, (9) catechol, and (10) tyramine. (B) Schematic diagram of CE-FSCV instrument (left). Electropherogram separation of tyramine, serotonin, octopamine, and dopamine in a single brain (Right). Cyclic voltammograms of each analyte are labeled at the corresponding peak. (C) Schematic diagram of a Drosophila hemolymph sampling setup (left). Electropherogram for fluorescamine labeled amino acids of wildtype adult fly hemolymph (right). (1) arginine, (2) citrulline, (3) tyrosine, (4) histidine, (5) glutamine, (6) asparagine and threonine, (7) alanine and serine, (8) taurine, (9) lysine, (10) glycine, (11) cysteine, (12) glutamate, (13) aspartate, and (14) unknown. Panel A modified from ref. [79], Panel B from [80], and Panel C from [85], all with permission from the American Chemical Society.

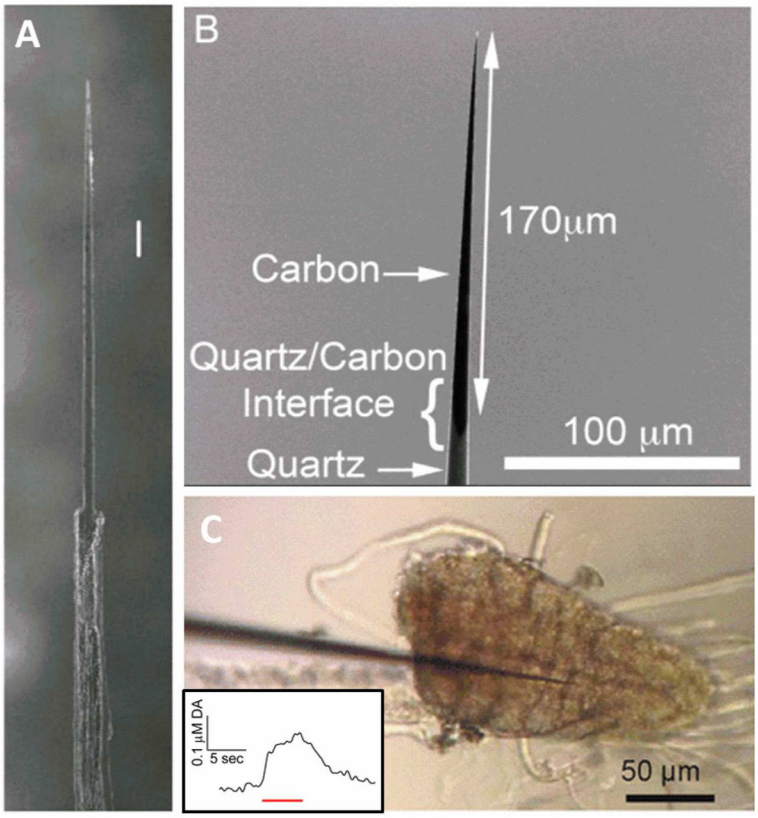

Figure 6.

Nanometer scale carbon electrodes. (A) Scanning electron microscopy (SEM) image of a flame-etched carbon electrode. Scale bar is 100 nm, tip is 1 μm. (B) SEM of carbon nanopipette electrode (CNPE, 50 nm tip diameter). (C) CNPE inserted into Drosophila VNC neuropils. Insert is dopamine after a 5 s continuous red light stimulation. Panel A is reprinted from ref. [94] and Panel B and C are reprinted and modified from ref. [96], both with permission of the American Chemical Society.