Characterization of optically and chemically stimulated neurotransmitter release in *Drosophila melanogaster*

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Abstract

Drosophila melanogaster is a popular model organism to study neurotransmission and neurological disorders. There is a wide range of genetic manipulation tools available for use in *Drosophila,* which has homologs for 75% of human disease genes. In addition, several fundamental neurological processes are conserved between the two species. *Drosophila* is therefore an excellent model organism for high throughput screening of genes involved in diseases and of drugs that can be used to treat the diseases. Since many neurological diseases are caused by changes in neurotransmitters, it is imperative to understand the fundamentals of neurochemistry and how it changes during a disease process. Electrochemical methods have long been used to measure neurotransmitters and their dynamics in the brain. Many neurotransmitters such as dopamine and octopamine are electroactive and can be measured directly on electrochemical sensors as described in this thesis.

Fast-scan cyclic voltammetry (FSCV) is a method that can provide rapid, sensitive, and selective measurements of neurotransmitters in the brain. This thesis describes new methods to stimulate endogenous dopamine release in *Drosophila* larval and adult CNS, and optimization of the FSCV waveform for measurements of endogenous octopamine in larvae. Chapter 2 describes acetylcholine, nicotine, and neonicotinoid stimulated dopamine release in

Drosophila larval VNC. Since acetylcholine acts on endogenous *Drosophila* receptor nAChR to stimulate dopamine release, this method can be used to study release in most *Drosophila* lines. The method was also used to make the first measurements of evoked dopamine release in adult *Drosophila* brains in our lab. Chapter 3 describes stimulated dopamine and dopamine tissue content measurements in the CNS of adult controls and Parkinson's disease model *Drosophila* with a knockdown of *Parkin* or *RNF11* with RNAi. We found that aging does not affect the concentration of stimulated dopamine release, or the tissue content in the CNS, but has an effect when stimulations are repeated at short intervals. The release in old adults declines significantly slower than in mid-age adults. This effect was lost in Parkinson's disease mutants suggesting that the mutations change dopamine dynamics in old adults. In Chapter 4, I describe optimization of the FSCV waveform for the detection of octopamine *in situ*. The new waveform uses a higher switching potential and a slower scan rate than the previously developed waveform for octopamine detection *in vitro*. With the new waveform, octopamine oxidation peak is detected away from the switching potential, where the background signal is most unstable. The waveform was used to measure and characterize light or ATP mediated octopamine release in *Drosophila* larval VNC. The methods described in this thesis enable measurements of stimulated dopamine release in most *Drosophila* larvae and adults, and stimulated octopamine release in larvae expressing transgenic ion

channels. Future studies can investigate the effects of disease or other mutations on the release and clearance of these neurotransmitters.

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1.1 Neurotransmission overview

1.1.1 Introduction

Brain cells, or neurons communicate with each other by means of electrical and chemical signals. Neurons are activated by an action potential, which occurs when there is an influx of sodium ions into the neuron making the inside of the neuron more positively charged than the extracellular space. Voltage-gated ion channels on the membrane of the neurons regulate ion flow across the cell and hence are responsible for activating and deactivating neurons by flow of mainly Na^+, K^+, Ca^{2+} and Cl^- ions. A neuron can pass a signal to another neuron by releasing neurotransmitters at the synapse, the space between two neurons. Major neurotransmitters in vertebrates are acetylcholine, dopamine, serotonin, epinephrine, norepinephrine, glutamate and GABA. Invertebrates also use the same neurotransmitters except they have tyramine and octopamine instead of epinephrine and norepinephrine.

Neurotransmitters are synthesized and stored in vesicles in neurons (Fig. 1.1). After neuronal firing, they are released from the presynaptic neuron into the synapse. They interact with receptor sites at the postsynaptic cells, which receive and propagate the signal. When a system of neurons, which is a group of neurons expressing one particular neurotransmitter are activated, a bigger volume of the brain rather than a single synapse is activated. This is called volume transmission and is caused when extracellular fluid containing the neurotransmitter diffuses to the extrasynaptic space.[1] Once released into the

synapse, the neurotransmitters can be metabolized, taken back up by the presynaptic cell through transporters (reuptake), or diffuse away.

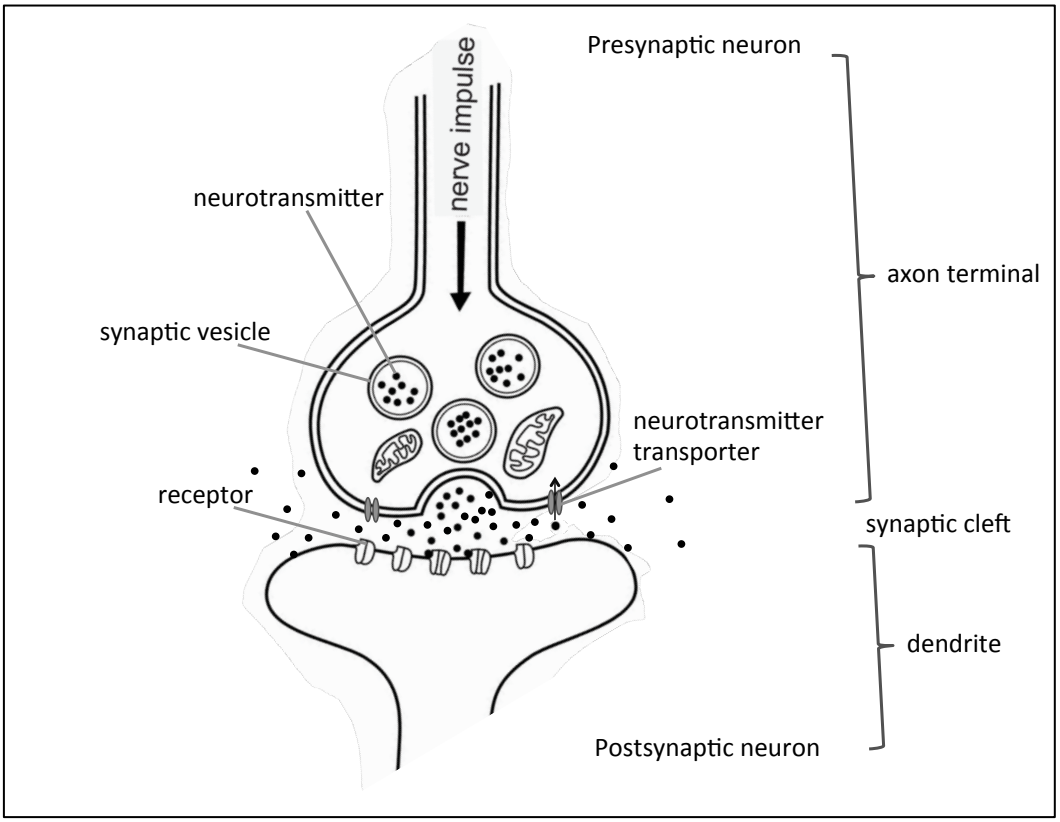

Figure 1.1. Schematic of neurotransmission. The presynaptic neuron synthesizes and stores neurotransmitters in synaptic vesicles. Upon neuronal firing, the vesicle fuses to the membrane releasing neurotransmitters into the synaptic cleft and activating the receptors on the postsynaptic membrane. Neurotransmitters can diffuse away from the synapse and activate neurons at extrasynaptic space, a process known as volume transmission.

Changes in one or more of these events of neurotransmission have been seen in several disorders. Alzheimer's disease is associated with acetylcholine deficiency.[2] Serotonin imbalance is found in depression[3] and impulsive behaviors,[4] and norepinephrine imbalance is seen with depression and ADHD.[5] There is a loss of dopaminergic neurons in the substantia nigra in Parkinson's

disease,[6] increased expression of dopamine transporters in ADHD[7, 8] and dopamine dysregulation in schizophrenia.[9]

As changes in the levels of neurotransmitters and their regulation can cause various disorders, it is important to understand how their levels change and in what ways they are regulated during a disorder. The concentration of neurotransmitters released in the synapse is in the nanomolar-micromolar range,[10, 11] and neurotransmitters are cleared rapidly in within milliseconds.[12, 13] Therefore, a fast and sensitive method is required to study neurotransmission. In addition, to study small brain structures, a method with high spatial resolution is needed. Finally, since the brain is a complex structure with many electroactive compounds, the method also needs to be selective. Using *Drosophila melanogaster* model organism, I have studied different neurotransmitters in the central nervous system of the insect with a rapid, sensitive and selective electrochemical method, fast-scan cyclic voltammetry (FSCV). This introduction will describe *Drosophila* as a model organism; neurotransmitters dopamine, octopamine and acetylcholine; fast-scan cyclic voltammetry (FSCV) at carbon fiber microelectrode (CFME); and optimization of FSCV for neurotransmitter detection.

1.2 *Drosophila melanogaster* - a model organism

The discovery of the white gene that causes the white eye phenotype in *Drosophila melanogaster*, or fruit flies, eventually led to discoveries of the role of

chromosome on heredity, and earned biologist Thomas Hunt Morgan the Novel Prize in Physiology.[14] Since this discovery in the early 1900s, *Drosophila* has been a popular model organism to study the effects of gene mutations from molecular[15] to behavioral[16] levels. A short life span, easy and low cost maintenance, fully sequenced genome and ease of genetic manipulation are some of the reasons for the popularity of *Drosophila* as a model organism.[17] Even though the fruit fly genome is much smaller than the human genome, many fundamental neurological processes are conserved between humans and *Drosophila.*[18] In addition, 75% of human disease genes have a homology in fruit flies, which makes it an important model organism to study human diseases.[19] Research in *Drosophila* has answered fundamental questions about sleep,[20, 21] Parkinson's disease,[22-24] and aggression[25] to give a few examples.

A wide variety of sophisticated genetic tools are available for *Drosophila*, more than any other multicellular organism.[26] The ability to create small changes in the genotype by adding or removing small portions of genes, or changing the levels of gene expression makes *Drosophila* an excellent model organism to understand the effect of small changes in the genome. Forward genetics has been a popular method to understand the genotype of diseases in *Drosophila*. It is a method where random mutations are created and the genotype that results in a particular phenotype is identified. Chemical mutations using ethyl methane sulfonate (EMS),[27] insertional mutations with transposable elements,[28] or expression of RNAi under GAL4-UAS have been used for forward genetics in

Drosophila.[29] The GAL4-UAS system was extensively used in my research and is described in detail below.

1.2.1 Galactosidase-4-upstream activating sequence (GAL4-UAS)

There are several genetic technologies available to precisely manipulate gene expression and study the downstream effects in *Drosophila.* Among the various toolkits available, the GAL4- upstream activating sequence (GAL4-UAS) system was the first technique used for the targeted expression of certain genes in specific cell or tissue type.[30] The GAL4-UAS system, described as "a fly geneticist's swiss army knife"[31] is a technique used to express specific proteins in a specific cell or tissue type. The gene *GAL4,* originally discovered in yeast, codes for the GAL4 protein. GAL4 can bind to and activate another gene, the upstream activating sequence (UAS), which in turn transcribes any gene under the control of UAS. As shown in Figure 1.2A, GAL4-UAS is a two-part activation system, which by crossing parent flies with GAL4 and UAS, can be used for restricted expression of genes in the nervous system of *Drosophila.* An example of using the system to express GFP in the dopaminergic neurons in a *Drosophila* brain is shown (Fig. 1.2B). Flies with GAL4 linked to dopamine synthesis enzyme tyrosine hydroxylase (TH), (*TH-GAL4),* are crossed with ones with *UAS-GFP.* In the offspring, only dopaminergic cells express GFP upon stimulation. *TH-GAL4; UAS-GFP Drosophila* brains show that there are different clusters of dopaminergic cells that project to different regions in the brain. The GAL4-UAS system can similarly be used to express proteins such as ion channels in specific

cell types such as dopaminergic cells. Here, the GAL4-UAS system has been used to express cation channels CsChrimson and $P2X_2$ (described below) in octopaminergic cells to study stimulated octopamine release.

GAL4-UAS system can also be used to knockdown specific proteins by using RNA interference (RNAi). RNAi is a process where cells silence an unwanted gene. The process is triggered when double-stranded RNA (dsRNA) is cleaved into smaller fragments called small interfering RNA (siRNA).[32] The siRNA bind to RNA-induced silencing complex (RISC), which separates the two strands, one of which is transported to the mRNA. The mRNA is hence silenced and gene is transcription is reduced.

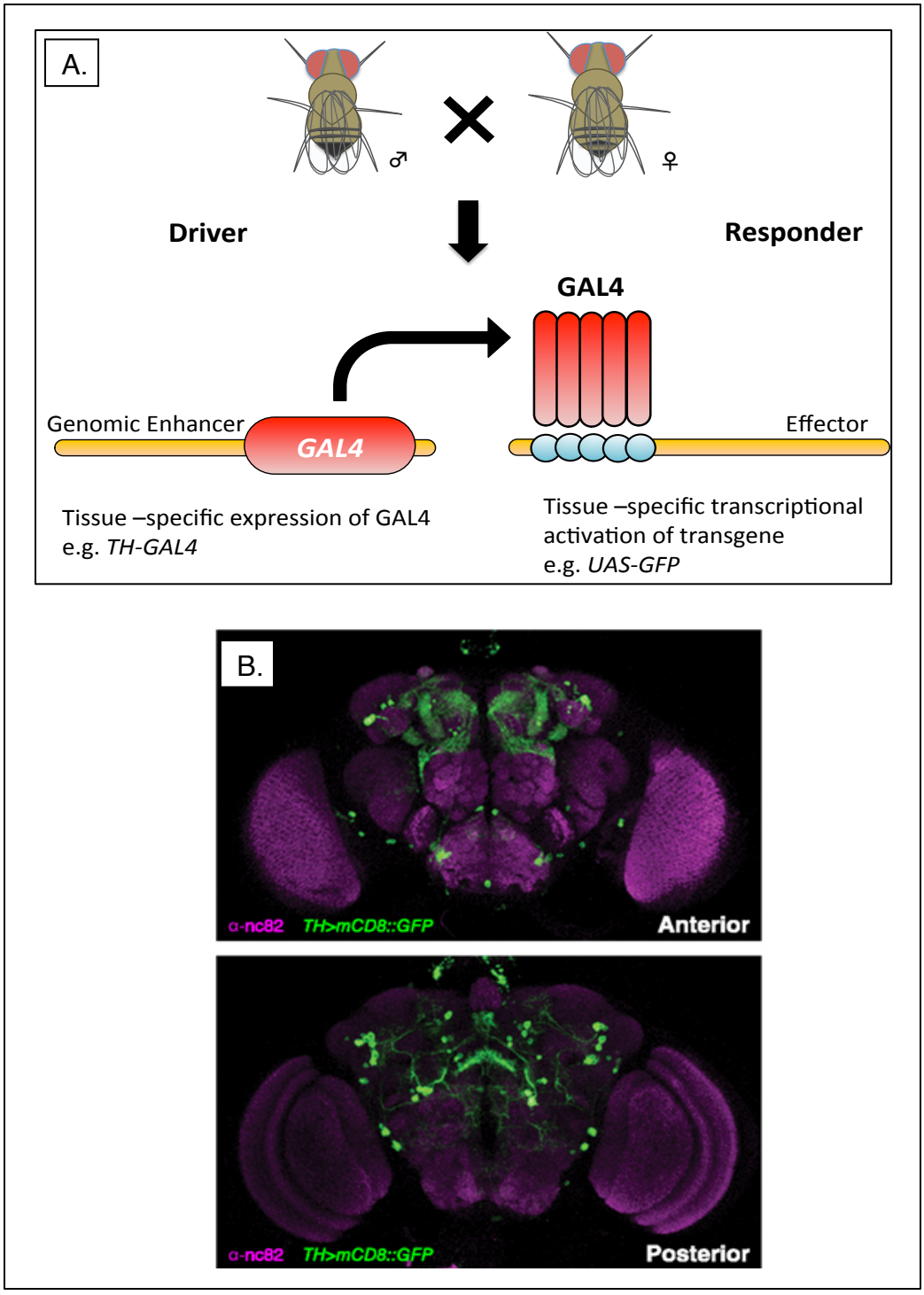

Figure 1.2. GAL4-UAS system. (A) When males carrying a GAL4 driver are crossed with virgin females carrying a UAS responder, the offspring contains both the driver and the responder. (B) Image of adult *Drosophila* brain expressing GFP on dopaminergic neurons (UAS-mCD8::GFP; TH1-Gal4) (reprinted from White K.E. et al., 2010).[2]

1.2.2 CsChrimson (CsChr) and P2X$_2$

CsChr is a non-selective cation channelrhodopsin activated by red light. CsChr has several advantages to a blue light sensitive channelrodopsin (ChR2) that was previously used in our lab.[33, 34] Red light has less energy, which causes less tissue damage and photoelectric effect.[35] It also travels deeper into tissue than blue light. CsChr also has faster turn-on, turn-off, and recovery kinetics allowing for more precise control of stimulation.[36] When cells expressing CsChr are stimulated by red light, the channel opens up allowing cations, mostly Na^+ into the cell, which depolarizes the cell causing an action potential and release of neurotransmitters.

P2X$_2$ belongs to a class of ligand-gated purinergic receptors that is activated by ATP.[37] Once bound to ATP, the channel opens up allowing cations to enter the cell and depolarizing it.[38] P2X$_2$ is also not found in *Drosophila* and can be genetically inserted into its genome.[39, 40] When ATP is puffed on for activation, P2X$_2$ channels bind to the molecules changing the channel confirmation, causing the channel to open and cells to depolarize. By using the GAL4-UAS system to express the channels CsChrimson (CsChr) or P2X$_2$ in octopaminergic cells, I have measured stimulated octopamine for the first time in *Drosophila* larva as described in Chapter 3 of this thesis.

1.2.3 Neurotransmitter measurement in life stages of Drosophila

The life cycle *Drosophila melanogaster* has four main stages – embryo, larva, pupa and adult (Fig. 1.3). The central nervous system (CNS) starts

developing at the embryonic stage where neurons and glial cells are present.[41] The CNS continues to grow and mature till adulthood and the neurotransmitter contents change throughout the life cycle.[42] Due to the short life span of *Drosophila,* it is an ideal model organism to study development and aging related disorders at different life stages.

Our lab has developed methods to study stimulated dopamine and serotonin release in *Drosophila* larvae. Blue light, red light or ATP stimulated dopamine and serotonin release have been measured and characterized in transgenic *Drosophila* larva. Stimulated dopamine concentrations, rate of dopamine clearance and *Km* values obtained in larval ventral nerve cord (VNC) are similar to electrically evoked release in mammals.[43, 44] Dopamine release and uptake parameters are different in different regions of the larval CNS.[35] Synthesis and reuptake of both dopamine and serotonin are needed to maintain the releasable pool of the neurotransmitters, and the dynamics of release and clearance of both neurotransmitters are similar to mammals.[39, 45]

Past research in our lab has established that *Drosophila* dopamine and serotonin neurotransmission are similar to mammals and that the model organism can be valuable in understanding the neurotransmitter systems better. However, we were not able to measure stimulated release in adult *Drosophila.* Chapter 3 of this thesis describes a method I have used to stimulated dopamine release in adult *Drosophila.*

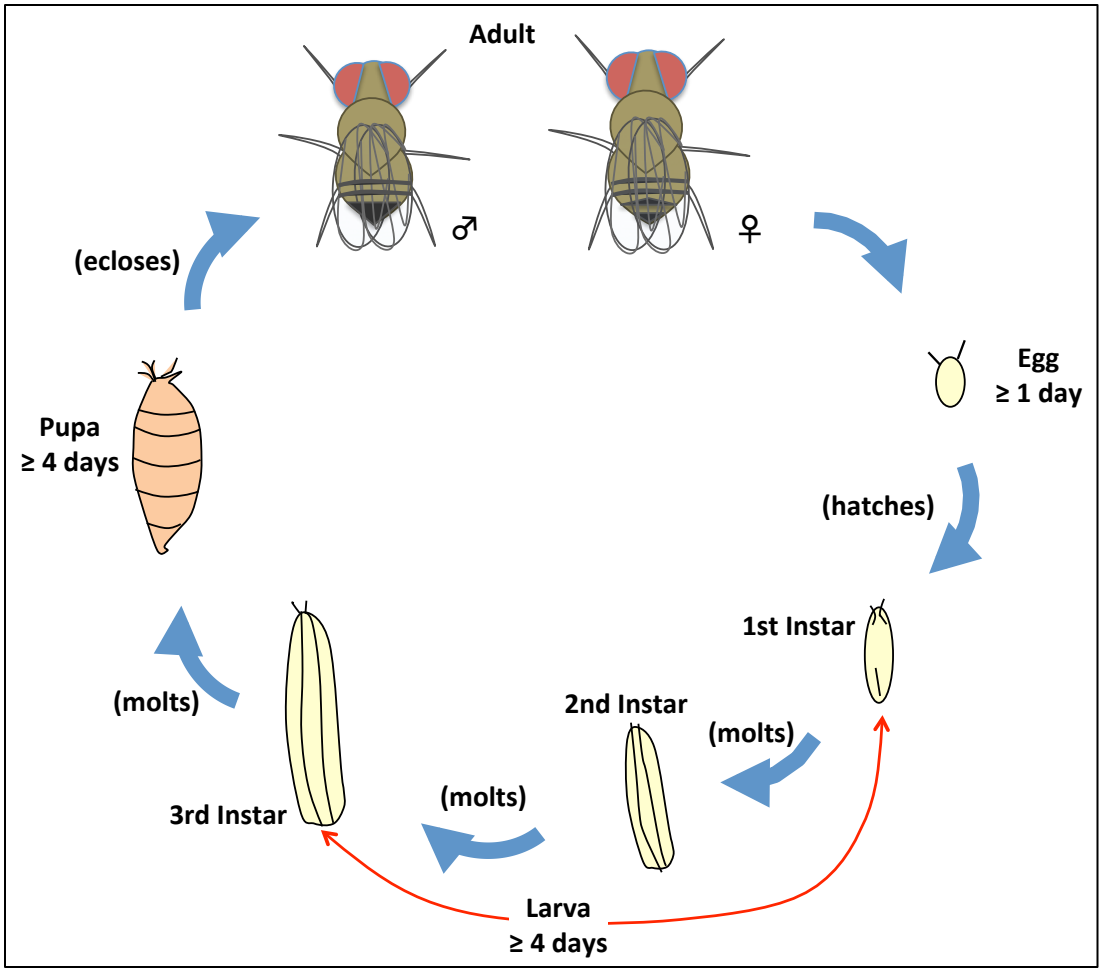

Figure 1.3. Life cycle of *Drosophila melanogaster*. The life cycle of *Drosophila* takes 9-10 days to complete at 25° C. Embryo hatch from fertilized eggs in ~1 day. The larval stage lasts ~4 days where the larvae grow in the food feeding generously. The third instar larva crawls out of the food to a dry part of the vial. It changes color becoming darker as it pupates. After ~4 day in the pupal stage the adult fly ecloses. The life cycle takes longer to complete at colder temperatures.

1.3 Neurotransmitters dopamine, octopamine and acetylcholine

Drosophila and humans share many common neurotransmitters – dopamine, serotonin, acetylcholine, glutamate and GABA. In addition to these, humans have epinephrine and norepinephrine, which are analogous to tyramine and octopamine in fruit flies (Fig. 1.4). Dopamine is a catecholamine neurotransmitter that plays a role in the reward pathways and in addiction.[46] It is

also involved in motor control[47] and is the major neurotransmitter affected in Parkinson's disease.[48] Many aspects of dopamine dynamics and signaling are conserved between humans and *Drosophila* and many drugs have the same effects in the dopaminergic system in both mammals and *Drosophila,* making it an excellent model organism for drug screening.[49] For example, tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (DDC) are the synthesis precursors for dopamine (Fig. 1.5) in both humans and *Drosophila,* and dopamine is packaged into vesicles through vesicular monoamine transporters (VMAT). Dopamine transporters (DAT) reuptakes released dopamine back into the presynaptic cells in both humans and *Drosophila.*

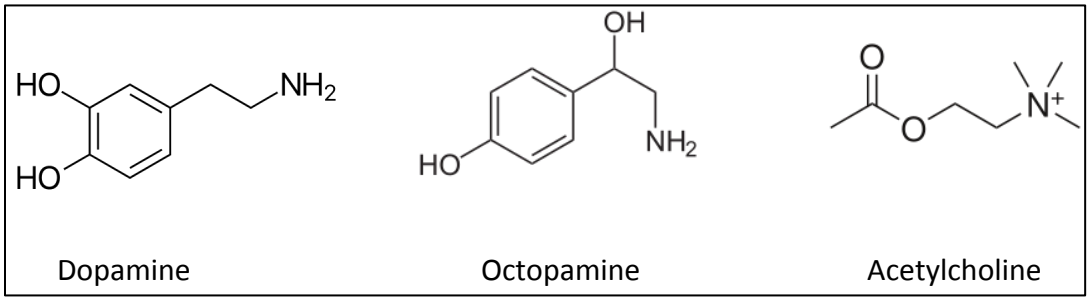

Figure 1.4. Chemical structures of neurotransmitters studied here.

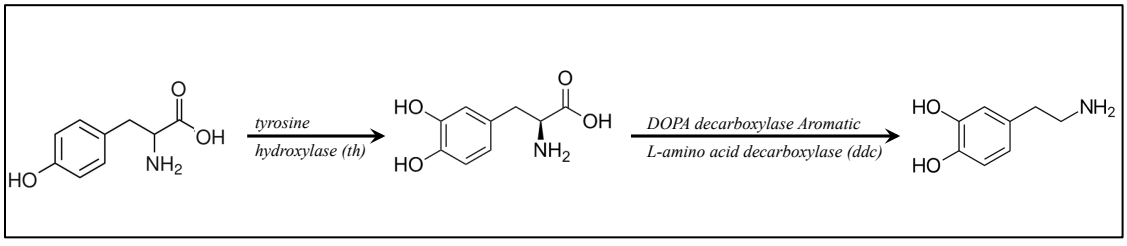

Figure 1.5. Synthesis pathway of dopamine. L-tyrosine is first converted to L-3,4-dihydroxyphenylalanine (L-dopa) by the enzyme tyrosine hydroxylase. L-dopa is then converted to dopamine by DOPA decarboxylase (DDC).

Due to the conserved structures between the two species, pharmacological agents have similar cellular effects in both humans and *Drosophila.* Studies of dopamine in *Drosophila* can identify potential drugs to be tested in the humans.[50] Current therapy for Parkinson's disease is symptomatic, and does not treat the underlying cause of the disease or slow its progression. Screening tests in *Drosophila* model of Parkinson's disease has found that celastrol and minocycline, which have antioxidant and anti-inflammatory properties slows dopaminergic cell loss.[51] We have also found that the protein RNF11, which is downregulated in Parkinson's disease increases stimulated dopamine release and clearance in *Drosophila* suggesting a compensatory mechanism for the cell loss.[52] Dopamine in *Drosophila* Parkinson's disease model has been further investigated as is described in Chapter 3 of this thesis.

Octopamine is a phenolamine that acts as a neurotransmitter, neurohormone, and neuromodulator in many invertebrates. It is analogous to norepinephrine in mammals and synthesized by homologous pathways.[53] In insects, the biosynthesis of octopamine is a two-step process where L-tyrosine is converted to tyramine by the enzyme tyrosine decarboxylase (TDC), and tyramine is converted to octopamine by tyramine beta hydroxylase (Tβh) (Fig. 1.6). There are two types of *Tdc* genes identified is *Drosophila melanogaster, Tdc1*, which is mostly expressed in non-neural tissues, and *Tdc2,* which is expressed in the CNS. Similar to dopamine, octopamine is packaged into

vesicles by VMAT. Unlike dopamine, however, there are no octopamine transporters identified in *Drosophila melanogaster*.

In insects, octopamine is involved in many important functions such as ovulation[54], olfactory learning and memory,[55] aggression,[56] and locomotion and grooming.[57] In mammals, octopamine is found in very small amounts and is suggested to play a role in modulating other neurotransmitters.[58] As it is a major invertebrate neurotransmitter and is suggested to play important roles in mammalian nervous system, I studied stimulated octopamine release in *Drosophila* larvae as described in Chapter 4.

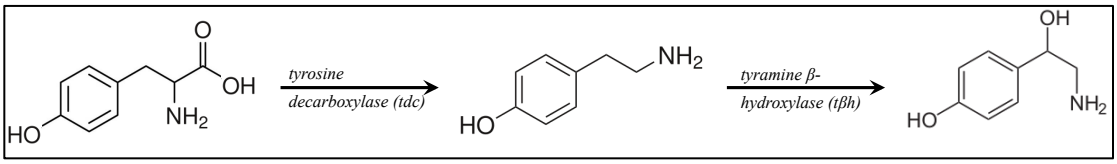

Figure 1.6. Octopamine synthesis pathway. L-tyrosine is first converted to tyramine by the enzyme tyrosine decarboxylase (tdc). Tyramine is then converted to octopamine by tyramine beta hydroxylase (tβh).

Acetylcholine is another major excitatory neurotransmitter in the CNS of insects. Cholinergic transmission is involved in mechanosensory responses,[59] wing movements[60] and in learning and memory formation.[61] Many insecticides like organophosphates, carbamates and neonicotinoids exert their toxic activity by disrupting the normal function of the cholinergic system. There are two broad classes of receptors acetylcholine acts on – nicotinic acetylcholine receptor (nAChR) and muscarinic acetylcholine receptor (mAchR). Nicotinic receptor, which are sensitive to nicotine and neonicotinoids are more abundant than muscarinic type in the nervous system of insects.[62, 63] nAChR activation causes

dopamine release in mammals; however, there are no such reports in *Drosophila.* As described in Chapter 2 of this thesis, I have discovered and characterized nAhcR mediated dopamine release in *Drosophila* larva.

1.3.1 Effect of insecticides on insect neurotransmitters

As mentioned above, many insecticides act on the nervous system of insects. Cocaine[64] and nicotine[65] are natural insecticides that potentiate neurotransmission and cause hyperactivity, spasmodic event and akinesia.[66] Responses to cocaine and nicotine is modulated by dopaminergic pathways in *Drosophila.*[66] Nicotine interacts with nAChRs, which are pentamers made of homologous or heterologous subunits.[67, 68] *Drosophila melanogaster* nAChRs consist of ten different subunits (Dα1 – Dα7 and Dβ1 – Dβ3).[69] Electrophysiological studies show fast transient synaptic currents mediated by nAChRs in cultured *Drosophila* embryonic neurons.[70] *Drosophila* Kenyon cells also exhibit an increase in intercellular [Ca^{2+}] in response to nAChR stimulation.[71]

Neonicotinoids are synthetic analogs of nicotine with lower mammalian toxicity and longer field stability. Similar to nicotine, they increase nerve transmission by interacting with acetylcholine receptors and increasing intercellular [Ca^{2+}].[72] They are a widely used class of insecticide, and covered 80% of total the insecticidal seed treatment market in 2008.[73] There are several types of neonicotinoids with different sensitivities towards different insects.[73] Insects can develop resistance against certain neonicotinoids, which in some cases has been related to nAChR subunit mutation(s). A single point mutation in

the β1 gene was found in 5 field populations of neonicotinoid imidacloprid resistant *Aphis gossypii.*[74] Mutations in the nAChR subunit genes in *Drosophila melanogaster* also created lines resistant to neonicotinoids.[75] Resistance to neonicotinoids is a threat to the global agriculture market and target-site investigation of the neonicotinoids can aid in developing efficient resistant management strategies.[73] *Drosophila* can be used to investigate nAChR subunit mutations that confer neonicotinoid resistance. This is particularly helpful since functional insect nAChRs are difficult to express in host cells.

1.4 Dopamine in Parkinson's disease

Parkinson's disease is a neurodegenerative disease that affects an estimated 10 million people in the world, and costs nearly $25 billion per year to the United States.[76, 77] It is a debilitating condition that mostly affects the elderly population. The disease is due to a loss of dopaminergic cell bodies in the substantia nigra of the brain, and reduced dopamine levels.[78-80] There is no treatment for Parkinson's disease, and medications that replace dopamine or are dopaminergic agonists only manage the symptoms. Major symptoms of Parkinson's disease are rest tremor, bradykinesia, rigidity and postural problems.[81] Although medications manage the symptoms, patients usually start developing severe side effects after 5 years of being on medication.[82] When Parkinson's disease is diagnosed, approximately 80% of the dopaminergic cells have already died. If the disease could be diagnosed earlier, the cell loss may be

prevented. However, currently there is no method for early disease diagnosis. Parkinson's disease is a complex condition that affects many neurotransmitter systems.[83-87] Therefore, it is imperative to understand how the different systems play a role in the disease. As genetic manipulations can be precisely controlled in *Drosophila,* it is an excellent model organism to study the cellular and molecular mechanisms of the disease to understand the cause and progression of the disease and to develop better medications.

There are several *Drosophila* lines that model Parkinson's disease. Effects of Parkinson's disease like mitochondria dysfunction,[88] muscle weakness,[89] and gait problems[90] are similar to phenotypes seen in *Drosophila* with Parkinson's disease mutations.[24, 91] A protein, RNF11, is downregulated in Parkinson's disease patients, and we found that in *Drosophila* it plays a role in dopamine neurotransmission.[52] In *Drosophila* that have a knockdown of the gene homologous to *RNF11*, there is an increase in stimulated dopamine release and clearance. This study was done in larval *Drosophila,* and Chapter 3 of this thesis describes study of Parkinson's disease model *Drosophila* at different life stages.

1.4.1 Parkinson disease model Drosophila melanogaster

Mutations in the *Parkin*,[92] PTEN-induced putative kinase 1 (*PINK1*),[93] and *alpha-synuclein*[94] genes have been linked to Parkinson's disease, but the mechanism of how the mutations lead to the disease is unclear. Recent work in *Drosophila* has provided evidence that both PINK1 and Parkin are involved in a pathway that regulates mitochondria dynamics[95] and its ability to cope with

oxidative stress.[96] Mutant *Drosophila* have symptoms such as muscle loss[24] and degeneration of dopaminergic neurons,[96] which are similar to humans with Parkinson's disease.[89, 97] A study in *Drosophila* also suggested a link between PINK1 and Parkin, which act on a linear pathway, and that Parkin functions downstream of PINK1.[98] More work on the Parkinson's disease model fruit flies can provide further details on the mechanism of the disease. I have studied stimulated dopamine release in *Drosophila* with Parkin mutation as described in Chapter 3.

Drosophila melanogaster has several advantages as a model organism. It has already answered many fundamental questions about neurotransmission and neurological disorders. Using a rapid electrochemical technique, FSCV, I have studied the real time dynamics of neurotransmitters in *Drosophila* to better understand neurochemical changes during insecticide resistance and in neurological disorder, Parkinson's disease.

1.5 Electrochemical detection of neurotransmitters

1.5.1 Introduction to electrochemistry

Electrochemistry is a branch of chemistry that studies the relation between electrical and chemical changes. An electrochemical reaction occurs when an applied current causes a chemical reaction, or when a chemical reaction produces a current; this thesis deals with the latter process. A redox reaction is an electrochemical reaction where there is a transfer of electrons; the species that loses electrons is oxidized and the species that gains electrons is reduced.

When a redox reaction occurs between neurotransmitters and the electrode, there is charge transfer at the interface of the electrode and the analyte. The charge is proportional to the number of moles of analyte reacted (Equation 1.1).

$$Q = nF\Delta N \qquad \text{(Equation 1.1)}$$

where Q = charge, n = number of electrons involved in the reaction, F = Faraday's constant and N = number of moles reacted.

Current is the rate of flow of charge (Equation 1.2), which can be measured and the number of moles of analyte calculated.

$$i = \frac{dQ}{dt} \qquad \text{(Equation 1.2)}$$

where i = current, Q = charge and t = time

1.5.2 Carbon fiber microelectrode for electrochemical detection

Carbon fibers are 5-10 µm in diameter and are mostly composed of carbon. They have several advantages including high tensile strength, high stiffness and high chemical resistance.[99, 100] Carbon fiber electrodes were first used for the detection of neurotransmitters and their metabolites with pulse polarography.[101] Since then, carbon fiber microelectrodes (CFMEs) have been a popular biosensor especially for the detection of neurotransmitters.[45, 102-105] A CFME is made of carbon fiber in insulated glass with protruding carbon length of 50 – 100 µm and a diameter of 7 µm. The small size of the CFME makes it ideal for measurements in specific brain areas as well as in small preparations such as *Drosophila* CNS. Carbon fibers also have advantages of biocompatibility and well characterized electrochemical properties.[102] Unmodified CFMEs have been used

for the detection of several neurotransmitters – dopamine, serotonin, norepinephrine, epinephrine, octopamine, tyramine, histamine, and adenosine.[45, 102-106] CFMEs can also be modified with enzymes to detect non-electroactive neurotransmitters like acetylcholine and glutamate.[107, 108]

1.5.3 Electrochemical detection of neurotransmitters at CFMEs

The main electrochemical methods used for measurements of neurotransmitters at CFMEs are amperometry,[109] chronoamperometry[110] and fast-scan cyclic voltammetry (FSCV).[34] CFMEs are well suited for real time detection of the rapidly changing levels of neurotransmitters in the brain. In amperometry, a constant potential is applied to the electrode and neurotransmitters are oxidized as they come in contact with the electrode. The technique has high temporal resolution but is not selective, which makes it not ideal for use in complex biological samples. Choronoamperometry is a potential step method and measures the ratio of oxidation to reduction currents at a given potential. For accurate measurements, it is important to keep the time between the potential steps long enough to separate faradaic current from charging current. Also, a large charging current is generated if the potential step is too large, making it different to detect faradaic current.[111] FSCV is a potential sweep method where the voltage is ramped up and down, and current response measured at different potentials. A background current must be subtracted to obtain a voltammogram. The shape of the voltammogram helps with compound identification as it has compound specific characteristics and peak potentials.

This chemical specificity gives FSCV a unique advantage over other electrochemical techniques. This thesis describes studies of neurotransmitters dopamine and octopamine with FSCV.

1.6 FSCV data collection and analysis

As mentioned in section 1.5.1, charge transfer at the interface of the electrode and analyte solution generates current, which is a Faradaic process. Any current generated by the movement of ions in solution without exchange of electrons at the interface is non-Faradaic (background current) and needs to be subtracted out from the Faradaic current. The main factors that increase the non-Faradaic charging current are increasing the electrode area or the scan rate. However, the charging current is stabilized after few minutes of waveform application, and can be subtracted out (background subtraction) to obtain just the Faradaic current. Before sample injection or stimulation *in vivo*, background current is collected for a few seconds.

Figure 1.7 describes FSCV detection of 1μM dopamine in vitro. Figure 1.7A shows the FSCV waveform used for dopamine detection. This waveform has been optimized for sensitive measurement of dopamine at CFMEs.[112, 113] Scanning at a high rate creates a large background current (Fig. 1.7B). The background current is stable and can be subtracted. Figure 1.7B shows the background currents with and without dopamine. A characteristic cyclic voltammogram (CV) (Fig. 1.7C) can be obtained after subtracting the background

current, which for dopamine has the oxidation peak around 0.6 V and reduction peak around -0.2 V versus Ag/AgCl electrode. Dopamine oxidizes around 0.2 V and the observed shift in the peak is a result of the high scan rate used in FSCV, which delays the time between the reaction and its detection. A false color plot is generated as multiple CVs are collected over time (Fig. 1.7D). A horizontal section of the color plot shows how the current changes with time at a particular voltage. Figure 1.7C (inset) shows the current vs. time plot at 0.6 V. Each electrode is calibrated *in vitro* and because current is linearly proportional to concentration, the concentration of neurotransmitters released upon stimulation *in vivo* can be calculated.

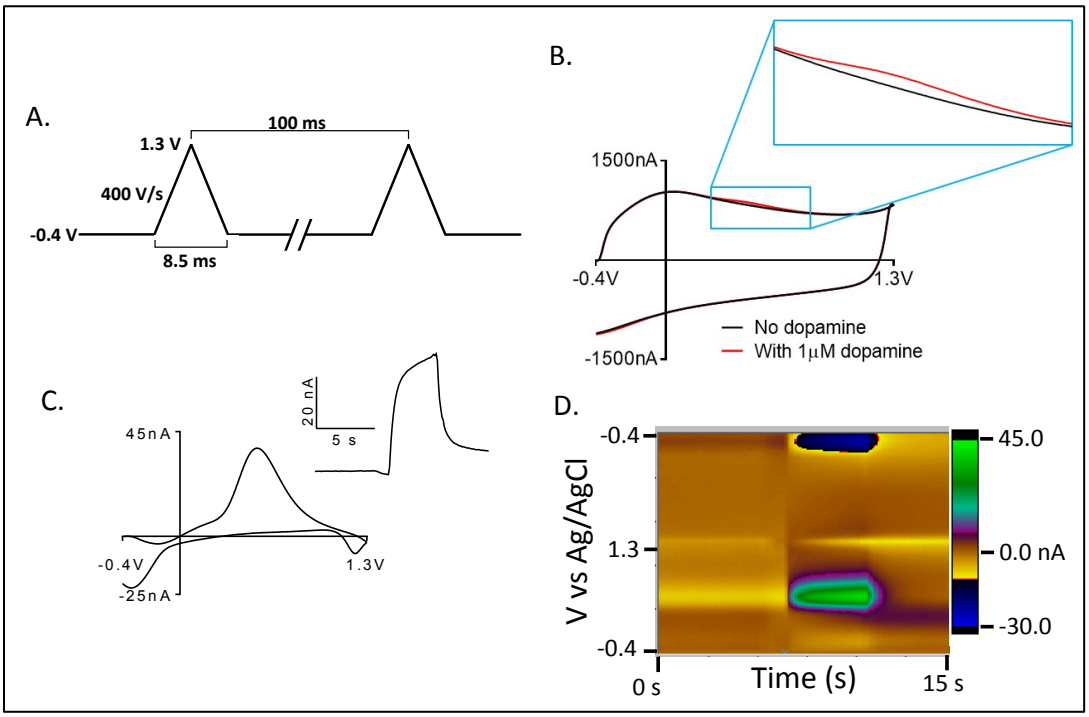

Figure 1.7. FSCV detection of dopamine *in vitro*. (A) FSCV waveform optimized for dopamine detection. (B) Scanning at a high rate creates a large background current (black line) which is stable. When dopamine is injected the current is slightly higher when dopamine oxidizes (red line). Since the background current is stable, it can be subtracted out to get the CV for dopamine. (C) Characteristic CV for dopamine with oxidation peak around 0.6 V and reduction peak around -0.2 V. The current vs. time plot at 0.6 V (inset). (D) False color plot with multiple background subtracted CVs. The color plot can be used to extract CVs at different times and current vs. time plots at different potentials. The large green oval is the time when dopamine was injected and oxidized. The blue oval at the top is the reduction of the dopamine oxidation product.

Properties of the electrode and measurement parameters affect the current as shown in Equation 1.3, which is for adsorption controlled redox reaction, and applies to all redox reactions described in this thesis.

$$i_p = \frac{n^2F^2 v A \Gamma^*}{4RT} \qquad \text{(Equation 1.3)}$$

where i_p = peak current, n = number of electrons involved in the reaction, F = Faraday's constant, v = scan rate, A = electrode surface area, Γ^* = moles of adsorbed reactant

1.6.1 Redox mechanisms for dopamine and octopamine

Dopamine oxidation is a two-electron process, where dopamine is oxidized to dopamine-o-quinone (DOQ) that can reduce back to dopamine.[114] The conversion of dopamine to DOQ is a reversible process, and some DOQ is reduced back to dopamine. DOQ can also cyclize to leucidopaminochrome, which can again oxidize to dopaminochrome. However, the cyclization rate for dopamine is low compared to other catecholamines.[115] Only the primary oxidation and reduction peaks are observed in the CV because of the slow cyclization and the low concentration of dopamine studied with FSCV.

Octopamine is a phenol with a different reaction mechanism. It is proposed that octopamine first goes through a one-step oxidation at the phenol

creating a radical.[104] The radical then reacts with another octopamine molecule to form a radical dimer. The dimer can further oxidize to create a product that can again react with more octopamine molecules to create a polymer. There are two oxidation peaks observed on octopamine CV, where the second peak lasts much longer than the first one. This long lasting secondary peak is thought to foul the electrode as subsequent measurements of octopamine with FSCV at standard dopamine waveform gave a decreasing response.

1.6.2 FSCV waveform optimization

The most popular waveform used with FSCV is triangular. Typical holding (lower) and switching (upper) potentials for dopamine measurements are -0.4 V and 1.3 V, respectively (Fig. 1.7A). The rate at which the voltage is ramped, the scan rate, is typically 400 V/s, and the waveform application frequency is typically 10 Hz for dopamine measurement. The negative holding potential of -0.4 V is kept for a relatively long time so the electrode can attract and adsorb the positively charged neurotransmitters.[116] When the voltage is ramped up and reaches the analyte's oxidation potential, the neurotransmitter is oxidized and when the voltage is ramped back down, it is reduced. The high scan rate and frequency allow rapid measurements and tracking of real time changes in neurotransmitter levels with high temporal resolution.

FSCV waveforms are usually optimized for the detection of specific analytes, such as serotonin,[117] adenosine,[118] tyramine and octopamine.[119] For example, the waveform used for octopamine detection *in vitro* was optimized to

be held at 0.1 V and ramped up to 1.3 V at 600 V/s. Octopamine oxidation reaction creates secondary products that can foul the electrode, and at 0.1 V holding potential the secondary peak was not observed. At high potentials, the carbon surface starts to fracture, which increases the surface area of the electrode, increasing analyte adsorption and reducing the limit of detection.[120] *In vitro* octopamine detection with 1.4 V switching potential gave a higher signal due to secondary oxidation product compared to the main product. Therefore, a switching potential of 1.3 was considered optimal. The current detected with FSCV increases with scan rate, and a rate of 600 V/s was chosen for *in vitro* detection of octopamine. This waveform, however, was not suitable for *in situ* detection of octopamine as the higher scan rate shifted the oxidation peak toward the switching potential. When used in tissues, the CV peaks often shift to higher potentials due to slowed electron transfer compared to *in vitro* measurements, hence skewing the peaks if they are too close to the switching potential.[121] Chapter 3 of this thesis describes optimization of the FSCV waveform for the detection of octopamine *in situ.* By using a waveform with a negative holding potential, a more positive switching potential and a slower scan rate, I could measure stable octopamine responses both *in vitro* and *in situ.*

Fast-scan cyclic voltammetry (FSCV) is a rapid, sensitive and selective technique that when used with carbon fiber microelectrodes (CFME) can provide high spatial resolution measurements of neurotransmitters. This thesis describes real time detection of neurotransmitters in *Drosophila melanogaster* central

nervous system with FSCV. In Chapter 2, I describe the first measurements of nicotine and acetylcholine stimulated dopamine release in *Drosophila* larvae. Nicotinic acetylcholine receptors mediate this release and subunit mutations control the extent to which neonicotinoid insecticides also stimulate release. In Chapter 3, I discuss the first measurements of acetylcholine stimulated dopamine release in adult *Drosophila,* and the study of stimulated dopamine and dopamine tissue content in Parkinson's disease model *Drosophila.* In Chapter 4, I report FSCV waveform optimization for the detection of octopamine. I also describe the use of this waveform for the first measurements of light and ATP stimulated octopamine in *Drosophila* larva.

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Chapter 2: Nicotinic acetylcholine receptor (nAChR) mediated dopamine release in larval *Drosophila melanogaster*

Chapter 2: Nicotinic acetylcholine receptor (nAChR) mediated dopamine release in larval *Drosophila melanogaster*

2.1 Abstract

Acetylcholine is an excitatory neurotransmitter in the central nervous system of insects and the nicotinic acetylcholine receptor (nAChR) is a target for neonicotinoid insecticides. Functional insect nAChRs are difficult to express in host cells, and hence difficult to study. In mammals, acetylcholine and nicotine evoke dopamine release, but the extent to which this mechanism is conserved in insects was unknown. In intact larval ventral nerve cords (VNCs), we studied dopamine evoked by acetylcholine, nicotine, or neonicotinoids. Using fast-scan cyclic voltammetry, we confirmed dopamine was measured by its cyclic voltammogram and also by feeding *Drosophila* the synthesis inhibitor, 3-iodotyrosine, which lowered the evoked dopamine response. Acetylcholine (1.8 pmol) evoked on average 0.43 +/- 0.04 µM dopamine. Dopamine release significantly decreased after incubation with α-bungarotoxin, demonstrating the release is mediated by nAChR, but atropine, a muscarinic AChR antagonist, had no effect. Nicotine ($t_{1/2}$ = 71 s) and the neonicotinoids nitenpyram and imidacloprid ($t_{1/2}$ = 86 s, 121 s respectively) also evoked dopamine release, which lasted longer than acetylcholine-stimulated release ($t_{1/2}$ = 19 s). Nicotine-stimulated dopamine was significantly lower in the presence of sodium channel blocker, tetrodotoxin, showing that the agonist acts on presynaptic nAChRs. *Drosophila* that have mutations in the nAChR subunit α1 or β2 have significantly lower neonicotinoid-stimulated release but no changes in nicotine-stimulated

release. This work demonstrates that nAChR agonists mediate dopamine release in *Drosophila* larval VNC and that mutations in nAChR subunits affect how insecticides stimulate dopamine release.

2.2 Introduction

Acetylcholine is a neurotransmitter in the central nervous system (CNS) of insects and the neuromuscular junction in vertebrates. It is the most abundant neurotransmitter in the CNS of insects,[1] where the cholinergic system mediates wing movements,[2] locomotion, learning, and memory.[3] The cholinergic system is also a target of insecticides such as organophosphates,[4] that inactivate acetylcholinesterase, and neonicotinoids,[5] that are acetylcholine receptor agonists.[6] Neonicotinoid insecticides work by overstimulating nicotinic acetylcholine receptor (nAChRs), which causes hyperexcitation, paralysis, and death of insects.[7] Acetylcholine receptors are either nicotine sensitive, muscarine sensitive (muscarinic acetylcholine receptor, mAchR), or of mixed nicotinic/muscarinic nature.[8] nAChRs are more abundant than mAChRs in the nervous system of insects, including *Drosophila melanogaster*. [9-11] Acetylcholine modulates neural activity in insects. For example, it causes depolarization, mediated by nAChRs, in cockroach giant interneurons[12] and elicits large excitatory currents and action potential bursts in the *Drosophila* larval central nervous system.[13] In rodents, nicotine increases dopamine release during phasic activity[14] and depletion of acetylcholine or the presence of an nAChR antagonist

decreases stimulated dopamine.[15] However, there are no studies of nAChR mediated dopamine release in insects.

Nicotinic acetylcholine receptors consist of five subunits that form a cation channel that is activated by acetylcholine interacting at the interface of two adjacent subunits.[16, 17] Benke and Breer were the first to suggest the existence of nAChRs in insects with differing affinity for α-bungarotoxin and different agonist sensitivities.[18] Since then, several different types of nAChRs subunit combinations have been identified which have different agonist affinity and activation kinetics.[19] Ten different subunits of nAChRs have been identified in *Drosophila,* Dα1 – Dα7 and Dβ1 – Dβ3[20]. α subunits contain a Cys-Cys pair, and are required for ligand binding.[21] Small changes in nAChR subunits affect neonicotinoid sensitivity, as a single point mutation in the β1 gene was identified in five field populations of *Aphis gossypii* resistant to the neonicotinoid imidacloprid.[22] While not a plant pest, *Drosophila melanogaster* is a popular model organism to study the insect nervous system and is particularly helpful to study structures such as insect nAChRs that are difficult to express in host cells.[23] *Drosophila* strains with mutations of Dα1, Dα2, or Dβ2 nAChR subunits are highly resistant to the neonicotinoids nitenpyram and imidacloprid, and mutations of different genes alter the resistance level.[24] Studies in *Drosophila* have revealed that the α5 subunit is involved in α-bungarotoxin sensitivity of nAChRs,[25] and that α6 subunit is essential for the insecticidal effect of spinosad.[26] The behavioral effects of nicotine on *Drosophila* are modulated by

dopamine,[27] so understanding how nAChR control dopamine release is critical for understanding the effects of neonicotinoids.

In this study, we characterized acetylcholine, nicotine, and neonicotinoid-stimulated dopamine release in *Drosophila* larval ventral nerve cord (VNC) for the first time. Our lab has pioneered measurements of dopamine in *Drosophila* using fast-scan cyclic voltammetry (FSCV) at implanted carbon-fiber microelectrodes (CFMEs), but previous experiments mainly used optogenetically-stimulated release.[28, 29] Here, we focus on release mediated by nAChRs and establish that acetylcholine, nicotine, and neonicotinoids cause dopamine release in the larval VNC. Longer duration release is evoked by nicotine and neonicotinoids than with acetylcholine. Stimulated release is sensitive to α-bungarotoxin (α-BTX) and tetrodotoxin (TTX) and is mediated by presynaptic nAChRs. Neonicotinoid-stimulated release is significantly lower in *Drosophila* nAChR subunit mutants that were previously found to have increased resistance to imidacloprid and nitenpyram. Thus, mutations that confer resistance to neonicotinoids in these strains also affect neonicotinoid-stimulated dopamine release. nAChR agonists stimulate dopamine release in *Drosophila* larval VNC; therefore, *Drosophila* can be used to study agonist sensitivities at mutated nAChRs subunits.

2.3 Results

2.3.1 Acetylcholine stimulates dopamine release in Drosophila melanogaster larval VNC

The effect of acetylcholine stimulation was studied in Canton S *Drosophila melanogaster* by pressure injection of acetylcholine into the neuropil of isolated larval VNC. A CFME and a pipet filled with 1 mM acetylcholine were placed about 10 µm apart (Fig. 2.1A); acetylcholine (1.8 nL droplet, or 1.8 pmol) was puffed into the larval VNC and the current response was measured with FSCV at the CFME. A trace of the concentration vs. time at the peak of oxidative potential (Fig. 2.1B) shows evoked dopamine changes over time while a trace of current vs. voltage, the cyclic voltammogram (inset), is a fingerprint of the molecule being detected. The cyclic voltammogram profile resembles that of dopamine with an oxidation peak around 0.6 V and reduction peak around -0.2 V.

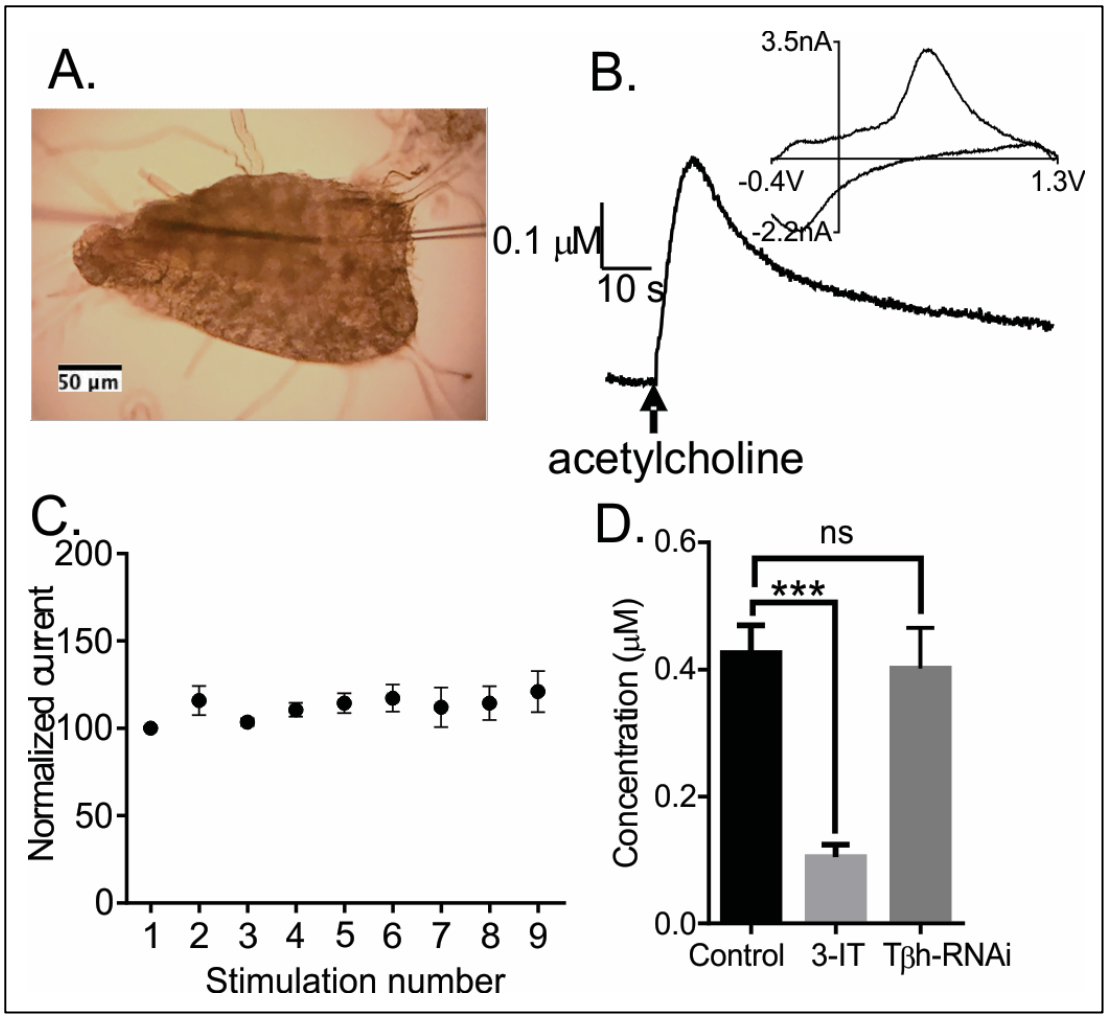

Figure 2.1. Acetylcholine-stimulated dopamine release. in *Drosophila melanogaster* ventral nerve cord (VNC). (A) Image showing electrode and stimulating pipet placed in VNC. (B) Acetylcholine (1.8 pmol) stimulated dopamine release measured with FSCV. The concentration vs time trace below shows changes in dopamine over time. The cyclic voltammogram confirms dopamine is detected. (C) When 1.8 pmol acetylcholine stimulations are repeated at five minute intervals, the current response is stable (no effect of stimulation, one-way ANOVA, $p = 0.66$, n = 6). (D) Acetylcholine stimulated dopamine release in larvae fed with dopamine synthesis inhibitor, 3-iodotyrosine, is significantly lower than control (unpaired t -test, $p = 0.0001$, n = 8-13), which verifies that the response is due to dopamine. Current response in larvae with octopamine synthesis enzyme knockdown (*Tdc2-GAL4;UAS-Tβh*^{RNAi}) is not significantly different than control (unpaired t-test, $p = 0.5678$, n = 5-13) suggesting that the response is not due to octopamine.

To test the stability of acetylcholine mediated release, repeated measurements were taken in the same VNC (Fig. 2.1C). When 1.8 pmol acetylcholine stimulation was repeated at 5 min intervals, the response was

stable for 9 stimulations as there was no significant difference in the current response measured over subsequent stimulations (one-way ANOVA, $p = 0.66$, n = 6).

To further verify that the response is due to dopamine release, larvae were fed with a dopamine synthesis inhibitor, 3-iodotyrosine (3-IT, 10 mg/mL in food for 48 hours). Acetylcholine evoked dopamine release (0.43 +/- 0.04 µM) was significantly lower in larvae fed 3-IT (0.10 +/- 0.02 µM, Fig. 2.1D) compared to control (unpaired t-test, $p = 0.0001$, n = 8-13). Acetylcholine-stimulated response was not significantly different in larvae with knockdown of octopamine synthesis using RNAi, *Tdc2-GAL4; UAS-RNAi*$^{T\beta H}$ (unpaired t-test, $p = 0.5678$, n = 5-13), which proves that the response is not due to octopamine.

2.3.2 Acetylcholine-stimulated release is mediated by nAChR and not mAchR

There are two types of acetylcholine receptors – nAChRs that are activated by nicotine and mAchRs that are activated by muscarine. To test the effect of nAChRs on acetylcholine-stimulated release, nAChRs were blocked by α-BTX. Figure 2.2A and C show that in the presence of 2 µM α-BTX ($K_d = 0.008$ and 1.14 nM for high and low binding sites on aphid membrane)[30, 31] the current is significantly lower than before the drug was applied (paired t-test, $p = 0.0002$, n = 8). To test the effect of mAchRs on acetylcholine-stimulated release, mAchRs were blocked by atropine. Figure 2.2B and C show that in the presence of 1 µM atropine[32] the current response is not significantly different before and after drug

application (paired t-test, $p = 0.6247$, n= 5). The results verify that acetylcholine-stimulated dopamine release is mediated by nAChRs, and not mAchRs, in *Drosophila* larval VNC. In *Drosophila*, there are two different subtypes of nAChR, α-BTX sensitive or insensitive,[23] so the lack of complete blockade by α-BTX suggests there could be α-BTX insensitive nAChRs in the larval VNC as well.

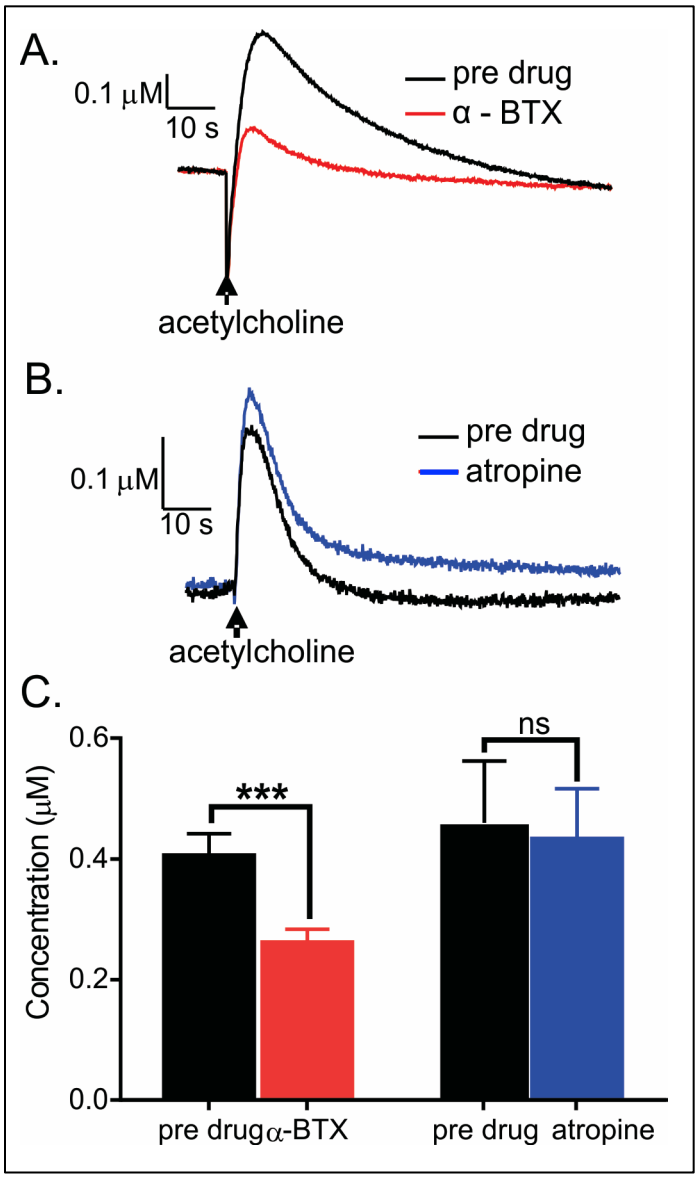

Figure 2.2. Acetylcholine-stimulated dopamine release is mediated by nAChR and not mAchR. (A) Example data of acetylcholine-stimulated dopamine release before and after bathing in nAChR inhibitor, 2 μM α-BTX. (B) Example data of acetylcholine-stimulated dopamine release before and after mAchR inhibitor, 1 μM atropine. (C) Averaged data for the drugs. The current response is significantly lower after α-BTX (paired t -test, p = 0.0002, n = 8) suggesting that the dopamine release is mediated by nAChRs, while there is no significant change after atropine (paired t -test, p = 0.6247, n = 5), which shows that the dopamine release is not mediated by mAchRs

2.3.3 Nicotine-stimulated dopamine release in Drosophila melanogaster larval VNC

Nicotine is a natural insecticide that competes with acetylcholine to act at nAChRs and disrupt their normal function. A nicotine puff (1.8 or 8.8 fmol) in the VNC resulted in dopamine release, as verified by the CV (Fig. 2.3A-B). A lower amount of nicotine was used than acetylcholine because nicotine has a higher binding affinity (K_D = 15 nM) than acetylcholine (K_D = 180 nM in rat brain membranes) and would be less tightly regulated, as it is not a natural neurotransmitter.[33, 34] The $t_{1/2}$ value, which is the time it takes to go from the peak current to half the current, is significantly higher for nicotine-stimulated release ($t_{1/2}$ = 71 ± 5 s for the 8.8 fmol stimulation) than for acetylcholine-stimulated release ($t_{1/2}$ = 19 ± 5 s 1.8 pmol stimulation) (one-way ANOVA multiple comparisons test, $p < 0.05$, n = 7). Similar to acetylcholine-stimulated release, the response was significantly lower in larvae fed 3-IT, verifying that it is due to dopamine (unpaired t-test, $p = 0.0345$, n = 5-6) (Fig. 2.3C). The nicotine-stimulated response was not significantly different in flies with knockdown of octopamine synthesis, *Tdc2-GAL4; UAS-RNAi*$^{T\beta H}$ (unpaired t-test, $p = 0.3250$, n = 4) (Fig. 2.3D). The sodium channel inhibitor TTX was used to examine whether release was exocytotic (Fig. 2.3E). The nicotine-stimulated response was significantly lower in the presence of TTX (paired t-test, $p = 0.0007$, n= 7). Blocking sodium channels inhibits the firing of action potentials, and therefore also inhibits exocytosis.

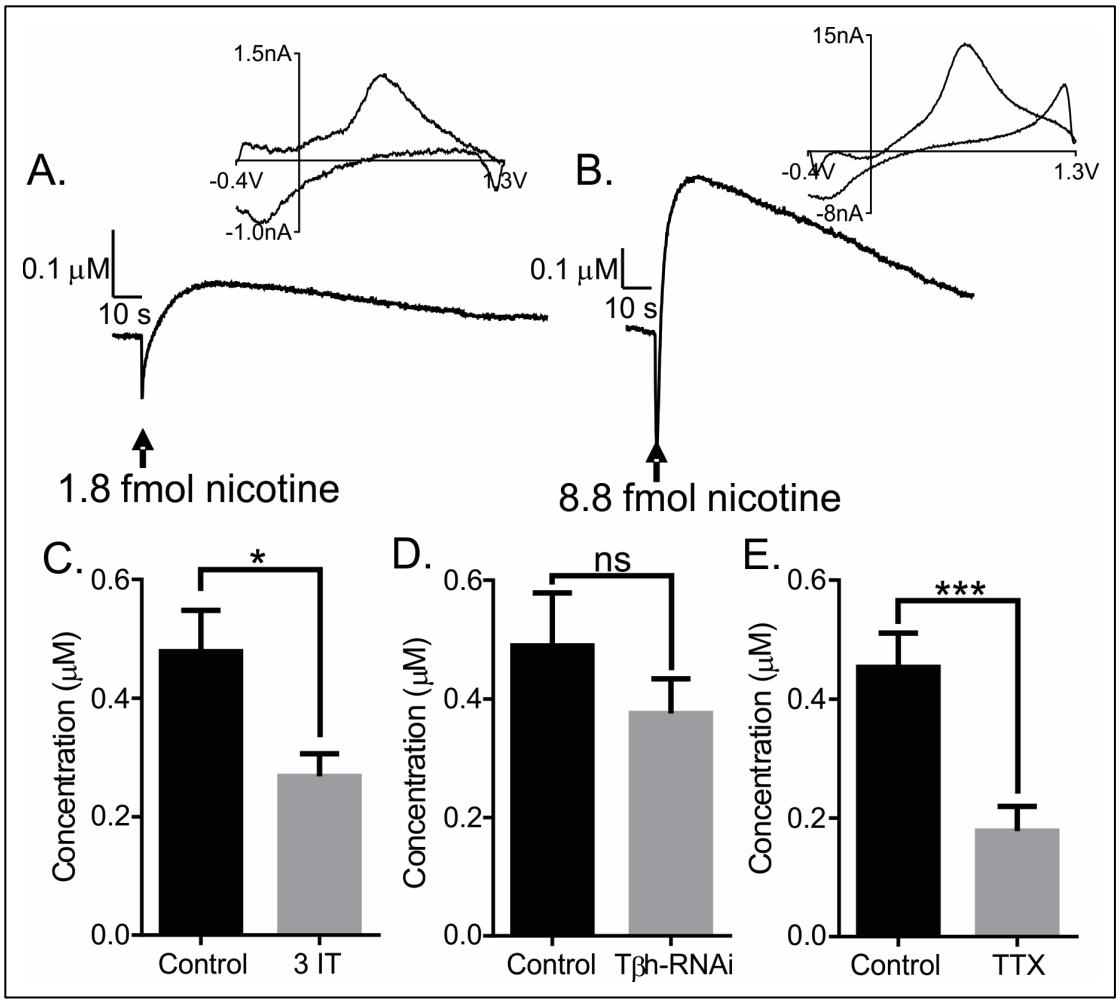

Figure 2.3. Nicotine stimulates dopamine release in the VNC, and the release is mediated by presynaptic nAChRs. (A-B) Current vs. time plot (bottom) and cyclic voltammogram (top) obtained upon nicotine stimulation in *Drosophila* larval VNC. (A) Example 1.8 fmol stimulation. (B) Example 8.8 fmol stimulation. (C) In flies fed dopamine synthesis inhibitor, 3 iodotyrosine, the current response is significantly lower (unpaired t -test, $p = 0.0345$, n = 5-6), which confirms that the response is due to dopamine. (D) Nicotine-stimulated response is not significantly different than control in flies with a knockdown of octopamine synthesis (*Tdc2-GAL4; UAS-RNAi*$^{T\beta H}$) (unpaired t-test, $p = 0.3250$, n = 4). (E) In the presence of sodium channel inhibitor, tetrodotoxin, the current response is significantly lower than control (paired t -test, $p = 0.0007$, n = 7), which shows that the release is mediated by presynaptic nAChRs.

Nicotine-stimulated release was measured repeatedly with 5 minute interstimulation times. The concentration of dopamine for each stimulation increases significantly and there is an effect of the amount of nicotine applied (Fig. 2.4A, 2-way ANOVA, significant effects of amount of nicotine and stimulation number, $p = 0.0011$ for nicotine concentration, $p < 0.0001$ for

stimulation number, and no significant interaction, $p = 0.97$). All of the higher dose stimulations elicited more dopamine than the lower dose stimulations (Fig. 2.4A, Sidak's post-test, $p < 0.05$ for all). The increase in release is better visualized when the data is normalized to the first stimulation (Fig. 2.4B). With the normalized data, there are again main effects of nicotine amount and stimulation number on evoked dopamine release (2-way ANOVA, $p < 0.0001$ for stimulation number and $p < 0.01$ for nicotine dose, interaction $p < 0.001$). Normalized responses with 1.8 fmol stimulation are significantly larger than the 8.8 fmol responses from 6^{th} to 9^{th} stimulations (Sidak's post-test, $p<0.05$). Sensitivity of nAChRs increased to nicotine upon subsequent stimulations, which is more apparent with lower amount of nicotine.

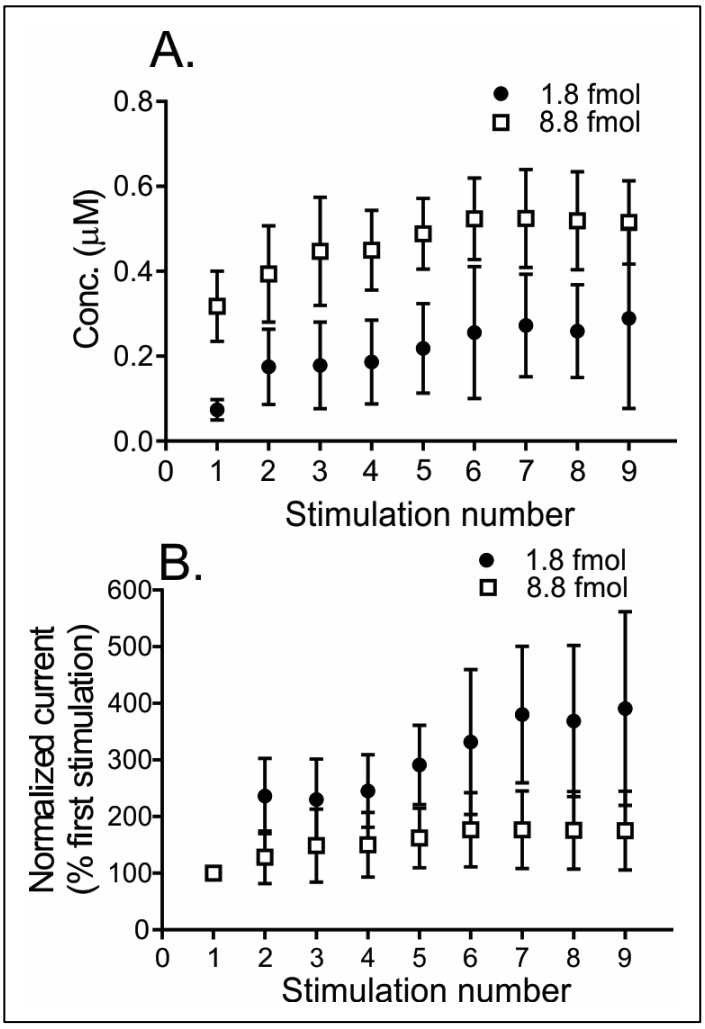

Figure 2.4. Repeated stimulations with nicotine. (A) The effect of repeated stimulations, 5 min apart, on the concentration of dopamine measured with 1.8 fmol or 8.8 fmol nicotine stimulation (2-way ANOVA, significant main effects of amount of nicotine and stimulation number, $p = 0.0011$ for nicotine concentration, $p < 0.0001$ for stimulation number, and no significant interaction, $p = 0.97$, n = 6). 8.8 fmol nicotine stimulation evokes more dopamine than 1.8 fmol nicotine (Sidak's post-test, $p < 0.05$ for all). (B) Normalized current for repeated stimulations, which also shows main effects of nicotine amount and stimulation number on the response (2-way ANOVA, $p < 0.0001$ for stimulation number and $p < 0.01$ for nicotine dose, interaction $p < 0.001$). There is a greater increase with 1.8 fmol than 8.8 fmol nicotine (Sidak's post-test), which suggests that the increase in sensitivity to nicotine occurs faster and reaches maximal level faster with higher amounts of nicotine.

2.3.4 Neonicotinoid-stimulated dopamine release in Drosophila melanogaster larval VNC

Two different neonicotinoids, nitenpyram and imidacloprid, were tested to determine the extent to which they evoked dopamine release. A higher amount

of nitenpyram (2.2 fmol) was used compared to imidacloprid (1.8 fmol) because imidacloprid is more potent in killing flies.[24] Stimulated dopamine release by both neonicotinoids (0.7 +/- 0.1 μM dopamine for nitenpyram and 0.32 +/- 0.04 μM for imidacloprid) had similar characteristics to nicotine-stimulated release (Fig. 2.5A and C). The cyclic voltammogram profile of the release was indicative of dopamine, and in larvae fed with 3-iodotyrosine, dopamine release was significantly lower with stimulation by nitenpyram (unpaired t-test, $p = 0.0284$, n = 5) or imidacloprid (unpaired t-test, $p = 0.0036$, n = 5) (Fig. 2.5B and D). The neonicotinoid-evoked response was longer lasting ($t_{1/2}$ = 86 +/- 7 s for nitenpyram and 121 +/- 20 s for imidacloprid) than acetylcholine-stimulated response ($t_{1/2}$ = 19 s, one-way ANOVA, $p < 0.05$, n = 7). The $t_{1/2}$ for imidacloprid-evoked response was also significantly higher than nicotine-stimulated response (one-way ANOVA, $p < 0.05$, n = 7).

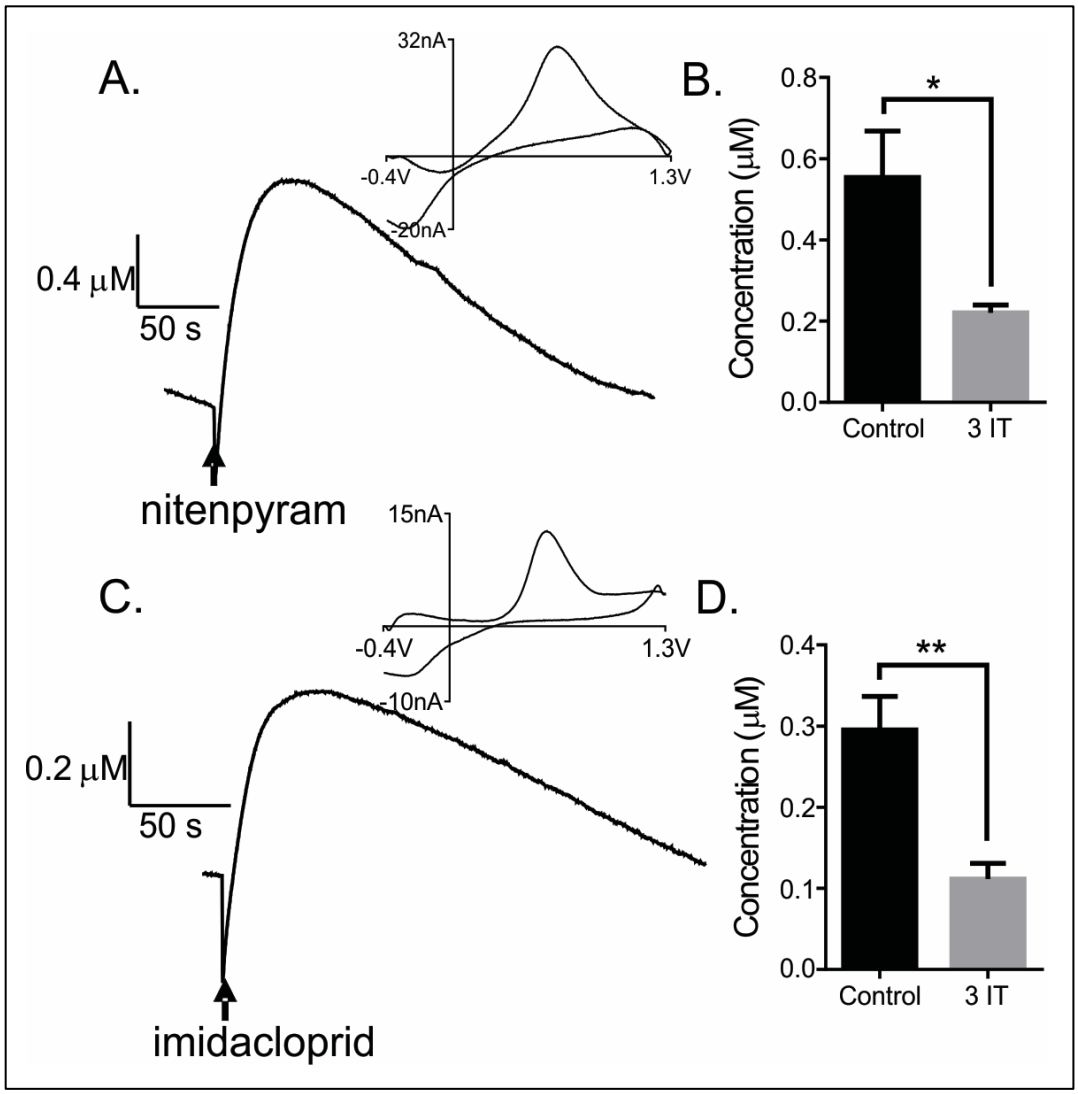

Figure 2.5. Neonicotinoids nitenpyram and imidacloprid stimulated release in *Drosophila melanogaster* ventral nerve cord (VNC). (A) 2.2 fmol nitenpyram and (C) 1.8 fmol imidacloprid evokes current response in the VNC, the cyclic voltammogram of which indicate dopamine. (B) Nitenpyram (unpaired t-test, $p = 0.0284$, n = 5) and (D) Imidacloprid-stimulated release is significantly lower in flies fed 3-iodotyrosine, confirming that the response is due to dopamine (unpaired t-test, $p = 0.0036$, n = 5).

2.3.5 Nicotine and neonicotinoid stimulated release in α and β nAChR subunit mutants

Perry et. al have identified mutations in the α and β subunits of *Drosophila* nAChR that confer reduced sensitivity to the neonicotinoids imidacloprid and nitenpyram.[24] To test if nicotine-stimulated release is affected by the mutations,

nicotine was puffed into the VNCs of *Drosophila* with α1 (EMS1) or β2 (EMS2) subunit mutations (Fig. 2.6A). The release upon nicotine stimulation was not significantly different (one-way ANOVA, $p = 0.7625$, n = 4), which suggests that effect of nicotine stimulation remains unchanged in these mutants.

To study the effect of neonicotinoid stimulations in the α1 (EMS1) and β2 (EMS2) mutants, nitenpyram or imidacloprid were puffed into the VNCs. The dopamine release in the mutant VNCs was significantly lower than control for both nitenpyram (one-way ANOVA, $p = 0.0010$, n = 4-6) and imidacloprid stimulation (one-way ANOVA, $p < 0.0001$, n = 5) (Fig. 2.6B and C), suggesting that the neonicotinoid binding sites are affected in these mutants.

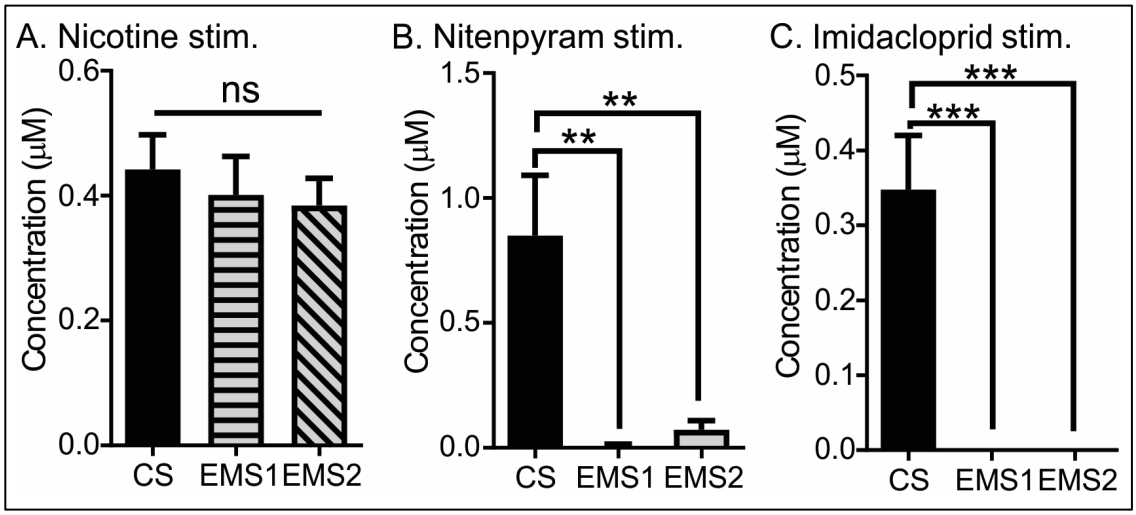

Figure 2.6. Effect of nAChR mutations on nicotine and neonicotinoid-stimulated response. (A) In flies with nAChR subunits α1 (EMS 1) or β2 (EMS 2) mutations, nicotine stimulation evokes release that is not significantly different than in wild type Canton S (CS) flies (one-way ANOVA, p = 0.7308, n = 4-7). (B) Nitenpyram (one-way ANOVA, p = 0.0010, n = 4-6) and (C) Imidacloprid-stimulated response is significantly lower in *Drosophila* with mutations in the nAChR subunits (one-way ANOVA, p < 0.0001, n = 5).

2.4 Discussion

Dopamine regulates behavioral responses to nicotine in *Drosophila*,[27] but neurochemical measurements of dopamine after nicotine stimulation had not been made. Here, we demonstrate for the first time that the stimulation of nAChRs causes dopamine release in *Drosophila melanogaster* larval VNC. These results are similar to findings in rodents that acetylcholine evokes dopamine.[27, 35] Acetylcholine, nicotine, and neonicotinoid insecticides evoked dopamine release in the *Drosophila* CNS and this response was mediated by nAChRs, and not mAchRs. All previous work in *Drosophila* larvae had used optogenetics or $P2X_2$ channels to stimulate dopamine release, which requires a channel to be expressed in specific neurons using genetics.[28, 29] Acetylcholine or nicotine stimulation evoke endogenous dopamine release, without needing to genetically modify the organism. Mutations in the subunits of the nAChRs affect neonicotinoid evoked dopamine release without changing the response to nicotine. Thus, dopamine measurements in *Drosophila* are an easy way to study the effects of nicotinic subunit mutations in causing excitatory responses.

2.4.1 Acetylcholine-stimulated dopamine release

Acetylcholine is the most abundant excitatory neurotransmitter in the CNS of insects [1] and plays a role in learning and memory formation.[3, 36] Dopamine is another major neurotransmitter that mediates many functions in *Drosophila,* including learning and memory[37] and modulates cholinergic transmission in *Drosophila* neuronal cultures.[38] Here, we discovered that acetylcholine

stimulation evokes a strong release of dopamine in the *Drosophila* larval VNC. The shape of the cyclic voltammogram and significantly lower response in *Drosophila* fed with 3-IT verify that the response is due to dopamine release. Because nAChR mediated octopamine release has been reported in adult fly brain preparations,[39] we tested flies with reduced octopamine synthesis and found no change in the evoked dopamine. The electrode placement for measuring from dopamine neurons is towards the thoracic region, away from the abdominal ganglia where octopamine has been measured.[40] Thus, this experiment was optimized for measuring acetylcholine receptor mediated dopamine release, although future studies could vary the electrode placement to target the abdominal segments of the neuropil to see if octopamine was released by acetylcholine.

Acetylcholine-stimulated dopamine release in flies is consistent with previous results in rodents, where it is well established that nAChRs are located presynaptically on dopamine neurons[41] and modulate dopamine release.[35] In rodents, mAchRs do not regulate dopamine release but nAChRs do.[15] Similarly, in flies, acetylcholine-stimulated release was significantly lower in the presence of nAChR inhibitor α-BTX, but not different with the mAchR inhibitor atropine. Blocking nAChRs and decreasing release implies that the nAChRs are presynaptically located on dopamine terminals and control dopamine release. The similarities in release indicate a conserved mechanism of dopamine

stimulation between flies and rodents, demonstrating that flies are a good model system to study nAChR mediated effects.

Stimulated dopamine release in *Drosophila* larval VNC has been previously studied with transgenic fruit flies that express either light or ATP sensitive ion channels in dopaminergic, serotoninergic, or octopaminergic cells.[29, 40, 42] Release using optogenetic stimulation was higher; a 7 s blue light stimulation evoked average of 810 +/- 60 nM dopamine, compared to the 430 +/- 40 nM with acetylcholine.[29] The $t_{1/2}$ for blue light-stimulated release was 7 s, which is shorter than 19 s observed for acetylcholine-stimulated release. The concentration of dopamine release and clearance time with acetylcholine were more similar to flies that were modified to express the $P2X_2$ channel, which was activated with exogenously applied ATP.[43] Puffing on the stimulus likely leads to longer activation and longer signals. While optogenetic methods allow specific stimulation because the channel is expressed in only one cell type, the disadvantage is that the flies must be genetically altered to express the channel using the GAL4-UAS system. To test the effects of other genetic mutations on dopamine release, it can be difficult to also express the mutation and the optogenetic channel in the same line. Thus, using acetylcholine, a natural neurotransmitter, is advantageous because dopamine release can be stimulated in any fly, including mutant flies, without needing genetic alterations to express an exogenous ion channel.

2.4.2 Nicotine-stimulated dopamine release

Nicotine is a natural insecticide that acts on nAChRs, overstimulating the nervous system of insects. The dopamine system is important for mediating the effects of nicotine in *Drosophila.*[27] For example, *Drosophila* exposed to volatilized nicotine show hyperactivity, spasmodic movements, and impaired ability to negatively geotax, and these behaviors are reduced by about 35% in flies fed with 3-IT.[27] We hypothesized that nicotine would elicit dopamine release through nAChRs and indeed, nicotine evoked dopamine release in a similar manner to acetylcholine. The sodium channel blocker, TTX, lowered nicotine- stimulated release significantly, demonstrating that release is exocytotic and suggesting that the nAChRs are presynaptic. One recent study found that simultaneous stimulation of two pathways: an acetylcholine pathway, mediated by nAChRs and a glutamatergic pathway, mediated by NMDA receptors, causes dopamine release in the mushroom bodies of adult *Drosophila.*[44] In that study, the dopamine neuron was postsynaptic to the two inputs, but here only acetylcholine is needed and there is no evidence for any activation other than simple presynaptic nAChRs.

There are some differences between acetylcholine and nicotine mediated dopamine release. The $t_{½}$ for acetylcholine mediated release is much smaller than that for nicotine mediated release. Acetylcholine, unlike nicotine, is a natural neurotransmitter that can be metabolized by acetylcholinesterase in the CNS of insects; hence, it would be rapidly cleared from the extracellular space.[45] Nicotine, on the other hand, is expected to be metabolized slower and mostly

cleared by diffusion, and give a longer lasting response.[46] The result agrees with previous observations of nicotine-stimulated release lasting much longer than acetylcholine-stimulated release in the rat substantia nigra and ventral tegmental area.[47] Similarly, the amount of acetylcholine (1 mM in pipette, 1.8 pmol injected) used for stimulations needs to be much higher than for nicotine (1-5 μM in pipette, 1.8 – 8.8 fmol injected), likely because acetylcholine is rapidly metabolized and has a lower binding affinity to nAChRs than nicotine.[33]

There are also differences in the stability of release with repeated stimulations for nicotine and acetylcholine. Acetylcholine-stimulated dopamine release was stable when repeated every 5 minutes, while nicotine-stimulated dopamine release increased with more stimulations. The percentage increase was largest for low amounts of nicotine applied, where the release never plateaued even when 9 stimulations were performed. With higher amounts of nicotine applied, the concentration of dopamine evoked was higher, but the percentage increase during subsequent stimulations was not as large and release plateaus after a few stimulations. Possible mechanisms for the increase in dopamine release include desensitization of nAChRs on GABA (i.e inhibitory) neurons[48] or upregulation of nAChRs.[49] Both of these mechanisms have been demonstrated in rats, but nicotine exposure to upregulate nAChRs was on the time course of 8-24 hours, not 1 hour, the time course of this experiment.[49] With 8.8 fmol nicotine applied, the desensitization and upregulation is expected to occur faster than with 1.8 fmol, where it occurs more gradually, so the increase

continues for more stimulations. Studies of behavioral sensitization to nicotine in rats have found that repeated doses are needed to induce sensitization with nicotine, and these doses should be delivered quickly (i.e. within seconds) and not once a day.[50] Indeed, with mammalian smoking addictions, inhalation is repeated multiple times[51] and insects may eat a plant multiple times, which would lead to sensitization compared to one single exposure. There are *Drosophila* models with increased nicotine sensitivity, and future studies can investigate if these flies are already sensitized and if they have a difference in release patterns with repeated stimulations.[52]

2.4.3 Neonicotinoid-stimulated release

Neonicotinoids are synthetic insecticides analogous in function to nicotine. They overstimulate and desensitize nAChRs, causing paralysis and death of insects.[7] Similar to nicotine, stimulations with neonicotinoids imidacloprid or nitenpyram caused a long lasting dopamine release compared to acetylcholine-stimulated response, and evoked dopamine was lower in larvae fed with 3-IT. However, the response for imidacloprid stimulation was even longer than the nicotine evoked response. A previous report found that neuronal firing evoked by imidacloprid fell more gradually than that evoked by nicotine.[53] Thus, imidacloprid may activate nAChRs longer than nicotine, which could be a factor for its effectiveness as an insecticide.

In mutant *Drosophila* lines that have increased resistance to nitenpyram and imidacloprid, neonicotinoid stimulation caused almost no dopamine release.

The resistant lines have a significantly lower mortality rate compared to wild type in the presence of the neonicotinoids in the growth medium.[24] These lines have mutations in the nAChR α1 (EMS 1) or β2 (EMS 2) subunits and both mutations were quite effective, as very little dopamine release was stimulated by either neonicotinoid.[24] While the EMS lines had reduced sensitivity to neonicotinoids, they maintained their sensitivity to nicotine, showing that the neonicotinoids likely have different binding sites at nAChRs. Indeed, previous studies demonstrated that nicotine and neonicotinoids have different interactions with acetylcholine binding proteins,[54] and different effects in resistant insects.[55] Neurochemical measurements in *Drosophila* are useful to study the downstream effects of nAChR subunit mutations on neurotransmitter release. Mutations in nAChR subunits confer resistance to insecticides, which is an important concern in agriculture. Our results demonstrate that dopamine stimulation is dramatically reduced in neonicotinoid resistant flies and other mutations could be studied to determine if they result in neonicotinoid resistance. Thus, dopamine measurements in *Drosophila* are useful for understanding the target specificity of neonicotinoid insecticides to different nAChRs.[55, 56]

2.5 Conclusions

We demonstrated for the first time that the nAChR agonists acetylcholine, nicotine, and neonicotinoids stimulate dopamine release in the *Drosophila* larval VNC. The release is mediated by presynaptic nAChRs, and not mAchRs, and is sensitive to tetrodotoxin, indicating release is exocytotic. Nicotine and

neonicotinoids stimulate dopamine release that lasts longer than acetylcholine-stimulated release, likely due to higher affinities and fewer mechanisms for their metabolism and clearance. Neonicotinoid-stimulated response is significantly lower in *Drosophila* strains that are resistant to the neonicotinoids and have mutations in α1 or β2 nAChR subunits. Nicotine-stimulated response, however, is not significantly different than controls in the mutant strains. Thus, *Drosophila* is an important model organism to study the effects of nAChR mutations or agonists on dopamine release and may yield important information about the pathways of acetylcholine regulation of dopamine release.

2.6 Experimental Section

2.6.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and solutions were prepared in Milli Q water (Millipore, Billerica, MA) unless noted otherwise. Electrode calibrations were performed in phosphate buffer solution (PBS; 131.25 mM NaCl, 3.0 mM KCl, 10.0 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.0 mM Na_2SO_4, and 1.2 mM $CaCl_2$) with pH adjusted to 7.4, which was made once a month and stored at 4 °C. To make the larval dissection buffer, 11.1 mM glucose and 5.3 mM trehalose were added to the PBS buffer on the day of the experiment. A 10 mM stock solution of dopamine was prepared in 0.1 M $HClO_4$ once a month and stored at 4 °C. Diluted dopamine solution for electrode calibration was prepared in PBS buffer the day of the experiment.

Acetylcholine chloride (1 mM) solution was prepared daily in PBS. 3-iodotyrosine was mixed with water and standard cornmeal food to make total concentration of 10 mg/ mL. Larvae were fed with 3 IT food for 2 days prior to experimentation. (-)-Nicotine ditartrate (Tocris Bioscience, Bristol, UK) was prepared in 1 µM or 5 µM solution in PBS on the day of experiment. Imidacloprid and nitenpyram were purchased from ChemService (West Chester, PA). Stocks of imidacloprid (1 mM in DMSO) and nitenpyram (1 mM in water) were diluted (to 1 µM for imidacloprid, 1.25 µM for nitenpyram) in PBS buffer.

Atropine (4 µM), alpha-bungarotoxin (8 µM) and tetrodotoxin (2 µM) were prepared in PBS buffer. To add drug to the VNC, 1 mL of the respective solution was added to the Petri dish that contained 3 mL of dissection buffer.

2.6.2 Drosophila and VNC Preparation

Drosophila melanogaster strains were obtained from Bloomington Stock Center: Canton S (stock #64349); GAL4 driver on octopaminergic/tyraminergernic neurons (Tdc2-GAL4, #9313); and UAS-RNAi on octopaminergic neurons, (UAS-RNAi$^{T\beta H}$, #27667). Strains resistant to neonicotinoids (EMS1 and EMS2) were obtained from Dr. Trent Perry at the University of Melbourne, Australia. *Drosophila melanogaster* stocks were maintained and crossed as described before.[28]

Larvae were dissected in modified PBS as previously described.[28] Briefly, the central nervous system was dissected out from a third instar larva using fine tweezers, the optic lobes were cut off, and the VNC was transferred to the lid of a

Petri dish with 3 mL of the dissection buffer in it. The opposite end of the VNC was cut with fine scissors to facilitate micropipette insertion.[57]

2.6.3 Electrochemical Setup and Data Analysis

CFMEs were fabricated with 7 µm diameter T-650 carbon fibers (Cytec Engineering Materials, West Patterson, NJ) in a 1.2 mm o.d. glass capillary (A-M systems, Carlsburg, WA) pulled to a tip. CFMEs were cut to 50–75 µm to form cylindrical electrodes. Data were collected with Dagan Chem-Clamp potentiostat (Dagan, Minneapolis, MN, $n = 0.01$ headstage), PCI 6711 and 6052 computer interface cards (National Instruments, Austin, TX), and a home-built breakout box. Tar Heel CV software (gift of Mark Wightman, University of North Carolina) was used for data collection and analysis. Electrodes were precalibrated with 1 µM dopamine in a flow cell.

The VNC and electrode were viewed under a 40X water immersion lens (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) and the electrode was inserted in the neuropil 4-5 segments from the cut end using a micromanipulator (Narshige N-MMN-1 and N-MMO-202ND). The potential was applied between the CFME and a Ag/AgCl reference electrode in the bath. A picospritzing pipet was made with the same glass capillary and vertical puller used to fabricate CFMEs. After being pulled, the pipet tips were trimmed. The pipet was filled with acetylcholine, nicotine or neonicotinoid and inserted into the VNC to pressure eject the agonists using Picospritzer III instrument (Parker Hannifin, Fairfield, NJ). The pipet was calibrated by measuring the diameter of liquid ejected in oil at a

set pressure and ejection time.

All statistics were performed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Significance was measured with a 95% confidence interval. Graphs are shown as mean ± standard error of the mean. For power analysis, mean acetylcholine-stimulated dopamine release of 0.45 μM, a 35% lower null hypothesis mean (0.29 μM) and standard deviation of 0.12 was used, which meant an n value of 5 was needed. Power analysis for nicotine-stimulated dopamine release used true mean of 0.50 μM, a 35% lower null hypothesis mean (0.33 μM) and standard deviation of 0.13, which also predicted a n=5 was needed.

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Chapter 3: Age-related changes in *Drosophila melanogaster* controls and *Parkin* or *RNF-11* mutants

Chapter 3: Age-related changes in *Drosophila melanogaster* controls and *Parkin* or *RNF-11* mutants

3.1. Abstract

Parkinson's disease is a neurodegenerative disorder characterized by loss of dopaminergic neurons in the substantia nigra region of the brain, most commonly in the elderly population. When the disease is diagnosed an approximately 80% of dopaminergic cells have already died. It is therefore important to understand early changes in neurochemistry so the disease can be identified earlier. *Drosophila melanogaster* Parkinson disease (PD) models have been developed to study molecular mechanisms of the disease. Using fast scan cyclic voltammetry (FSCV) we studied age-related changes in acetylcholine-stimulated dopamine release and dopamine tissue content in the CNS for control flies and a fly model Parkinson disease – *Parkin-RNAi.* We also tested a potential genetic target for PD treatment, *RNF11-RNAi*. We found a significant effect of age on evoked dopamine current with repeated short interval stimulations, as old flies had significantly less decrease of dopamine than mid-age flies. This effect of age was not seen in the Parkinson's disease model flies suggesting that these flies lose the ability to maintain dopamine signaling. There was a significant effect of age on the tissue content of *RNF11-RNAi* flies where old flies had a significantly higher dopamine tissue content than younger flies. There was also an effect of genotype when repeated stimulations were compared in old flies of the different genotype. This work shows that the Parkinson disease mutation

does not have a dramatic effect on dopamine levels but that there are subtle changes in the ability to maintain release for repeated stimulations.

3.2. Introduction

Parkinson's disease is a neurodegenerative disorder that affects over 10 million people worldwide.[1] It is a debilitating disease that affects mood, memory, sleep and motor functions. The disease is most common in people over the age of 50, and the incidence of the disease increases with age.[2] Most cases of Parkinson's disease are sporadic and causes of the disease are not well known although genetic,[3] environmental,[4] and interaction of environmental-genetic factors[5, 6] increases the likelihood of developing the disease. Mutations in several genes such as *α-synuclein*[7], *Parkin*[8], *PINK1*[6] and *LRRK2*[9] are linked to Parkinson's disease. Although the mutations are in different genes, the cellular effects of the disease are the same – loss of dopaminergic cells in the substantia nigra pars compacta in the midbrain.[10] The symptoms of the disease develop when up to 80 % of dopamine-producing cells have already died.[11] The disease is diagnosed based on symptoms and responsiveness to Parkinson's drug treatment and there is no good method for early disease diagnosis.[12] The treatment options available are only symptomatic, and there is no cure or disease modifying treatment available. Development of neuroprotective treatments has been slow mainly due to the limited knowledge of the molecular mechanism of the disease. Better understanding of the mechanism of dopaminergic cell loss

and disease biomarkers is needed for better drug development and early diagnosis of the disease. Model organisms like *Caenorhabditis elegans* and *Drosophila melanogaster* have been used for high throughput genetic screening for better understanding of the molecular mechanisms of neurodegenerative diseases.[13, 14] However, the changes in neurochemistry caused by these genetic mutations in Parkinson disease are largely unknown.

Drosophila melanogaster has been extensively studied as model organism for Parkinson's disease.[15-18] Fundamental aspects of dopamine synthesis, release, and signaling processes are conserved between humans and *Drosophila.*[19] This makes it an excellent model organism to study the cellular, molecular, and neurochemical changes that accompany dopaminergic cell loss in Parkinson's disease. There are several genetic manipulation tools available for use in *Drosophila* that have been used to create mutations in *Drosophila* homologs for *Parkin, PINK1, LRR2* and other Parkinson's disease genes. Parkinson's disease model *Drosophila* develop phenotype such as loss of mitochondrial integrity, muscle loss,[20] locomotor defects, abnormal wing posture and defects in climbing.[18] Similar symptoms such as mitochondria dysfunction,[21] muscle weakness,[22] and gait and postural problems[23] occurs in patients with Parkinson's disease. In *Drosophila*, however, there is conflicting evidence on how much the different Parkinson's disease mutations affect the dopaminergic cells, as some *Parkin* mutants do not show a loss of dopaminergic cells bodies.[24, 25] Some *Parkin* mutants do have a degeneration of a subset of dopaminergic

neurons such as the PPL1 or PPL2ab region and a decrease in dopamine synthesis enzyme tyrosine hydroxylase (TH).[14, 16, 26] Parkinson's disease patients also have similar cellular effects.[27] *Drosophila* can, therefore, be used to understand the neurochemical mechanism of Parkinson's disease.

RNF11 is expressed in neurons and is another protein linked to Parkinson's disease.[28] There is a decreased expression of *RNF11* in dopaminergic cells of Parkinson's disease patients, and *RNF11* knockdown increases protection of dopaminergic cells in 6-OHDA Parkinson's disease models.[29, 30] We have shown that a knockdown of *RNF11* gene using RNAi causes an increase in stimulated dopamine release and clearance in *Drosophila* larvae.[31] However, the extent to which RNF11 affects dopamine signaling in adults is unknown.

Here, I have studied acetylcholine-stimulated dopamine release in adult *Drosophila* at different ages, including control Canton S flies and flies with *Parkin-RNAi* or *RNF11-RNAi* mutations. Previously, acetylcholine-stimulated dopamine release was measured in *Drosophila* larval VNC, but here acetylcholine-stimulated dopamine release in adult *Drosophila* brains is measured for the first time. The measurements are made with FSCV at CFMEs and the small size of CFME allowed measurements of dopamine in the central complex region of *Drosophila* brain. The changes in tissue content of dopamine with age were also investigated in these lines. There is a significant difference in dopamine tissue content in adult *Drosophila* with the *RNF11-RNAi* mutation as *Drosophila* age. I

also found that dopamine releasable pool replenishes faster in aging control, but not in the *Parkin* or *RNF11* knockdown lines. There are conflicting evidence on the effects of different *Parkinson's* disease mutations in dopamine levels in *Drosophila* brains,[24, 25, 32] and the results here indicate that *Parkin-RNAi* or *RNF11-RNAi* mutations do affect how fast the releasable pool of dopamine restores in old-age flies.

3.3. Results

3.3.1 Acetylcholine-stimulated dopamine release in Drosophila melanogaster adult brain

Acetylcholine-stimulated dopamine release was discovered in *Drosophila* larval VNC, and here we measured stimulated release in adult *Drosophila* brains for the first time. A CFME was placed 50 µm deep into the center of the brain to target the central complex (Fig 3.1A). A pipet filled with 5 mM acetylcholine was placed about 10 µm from the CFME. When acetylcholine was puffed into the brain, a current response for dopamine was measured with FSCV (Fig 3.1B). Acetylcholine is not electroactive and is not measured. A horizontal slice of the color plot was taken to see how dopamine concentration changes with time (Fig 3.1B center). A vertical slice of the color plot was taken at maximal current, which shows the CV has the fingerprint of dopamine (Fig 3.1B inset).

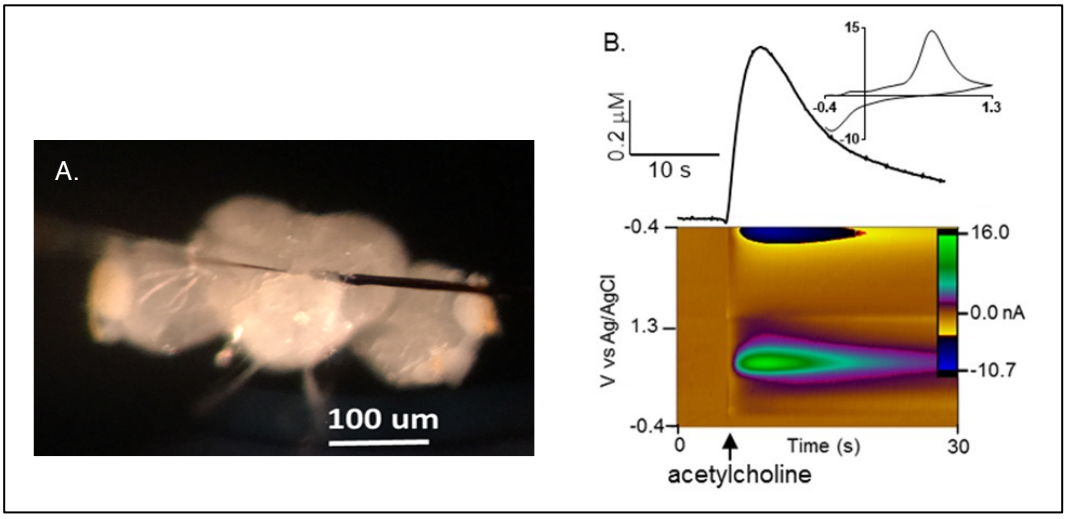

Figure 3.1. Acetylcholine-stimulated dopamine in the central complex region of *Drosophila melanogaster* brain. (A) Image of *Drosophila* brain with electrode coming from left and placed on the central complex. The image also shows stimulating pipet coming from the right. (B) The false color plot (bottom) and concentration vs. time plot (center) show how current changes over time. When 10 pmol acetylcholine is puffed on at 5 mins, there is a signal. The green oval in the center is oxidation current. When a vertical slice of the color plot is taken at peak current time a CV is obtained (inset). The electrochemical fingerprint of the CV shows that the current is due to dopamine.

The concentration of dopamine released with different amounts of acetylcholine stimulation was measured (Fig. 3.2). There is a significant effect of the amount of acetylcholine stimulation on evoked dopamine concentration (one-way ANOVA, $p = 0.0004$, n = 6). The concentration of dopamine increases with increasing acetylcholine stimulation, and plateaus at 10 pmol acetylcholine after which it is not significantly different up to 40 pmol (one-way ANOVA Tukey's multiple comparisons test, $p < 0.05$). Therefore, to measure maximal release 10 pmol acetylcholine stimulation was used in adult *Drosophila* brains.

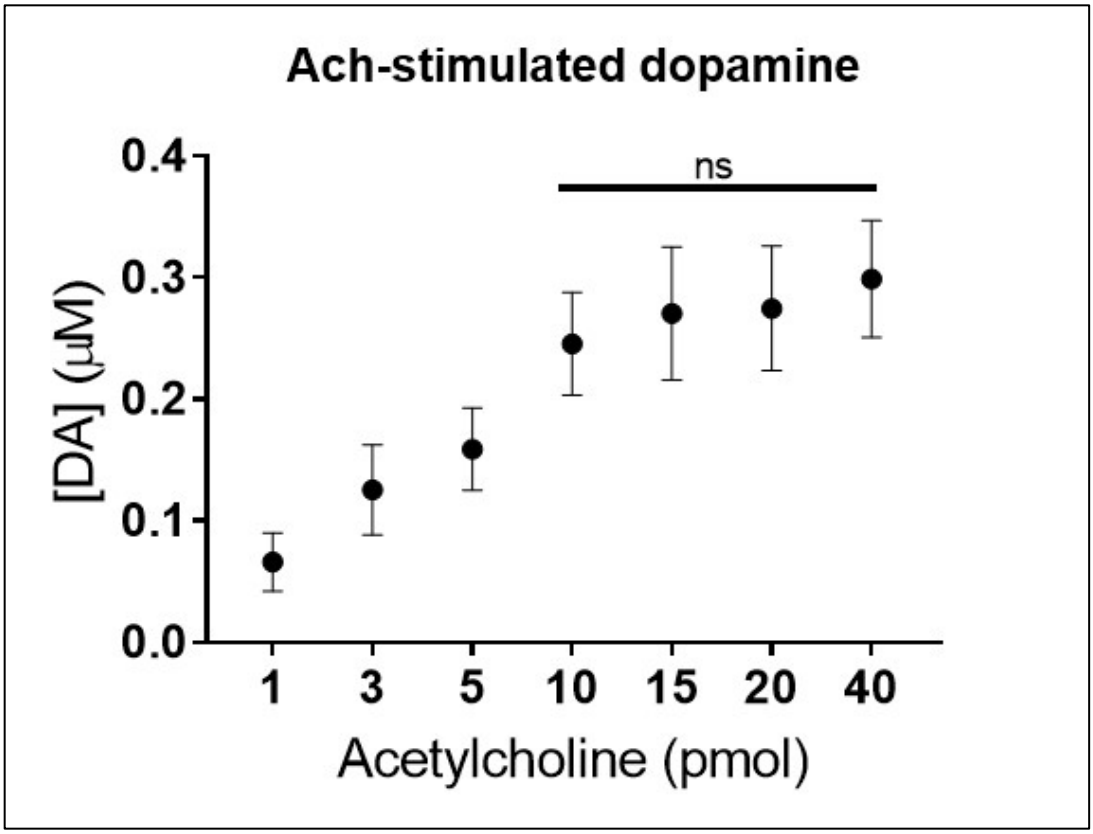

Figure 3.2. Dopamine release with increasing acetylcholine stimulation. There is a significant effect of the amount of acetylcholine stimulation as the concentration of evoked dopamine increases with increasing acetylcholine stimulation (one-way ANOVA, $p = 0.0004$, n = 6). The response plateaus at 10 pmol as there is no significant increased in evoked dopamine concentration at higher than10 pmol stimulation (one-way ANOVA, Tukey's multiple comparisons test, $p < 0.05$).

3.3.2 Stimulated dopamine and dopamine tissue content with age

Levels of dopamine synthesis enzyme (TH) in the striatum decrease with age in humans and correlate with dopaminergic cells loss in the substantia nigra, where dopamine is synthesized.[33, 34] We hypothesized that similar to human brains, *Drosophila* CNS will have a significant reduction in dopamine with age. We studied stimulated dopamine release and dopamine tissue content at different ages in adult *Drosophila*.

Acetylcholine stimulated dopamine release in the central complex region was studied at three different ages – young adult (1-5 day old), mid-age adult (20-25 day) and old adult (40-45 day). Figure 3.3 A-C show the color plots, concentration vs. time plots, and the CVs for stimulated release at different ages. The concentration of dopamine released with 10 pmol acetylcholine stimulation was not significantly different between the age groups (one-way ANOVA, p = 0.6474, n = 4-6) (Fig. 3.3D). In contrast to our hypothesis, the release was slightly higher as *Drosophila* aged, although the result was not significantly different. Dopamine tissue content in the CNS was also not significantly different at the different ages (one-way ANOVA, p = 0.0758, n = 5-8) (Fig. 3.3E).

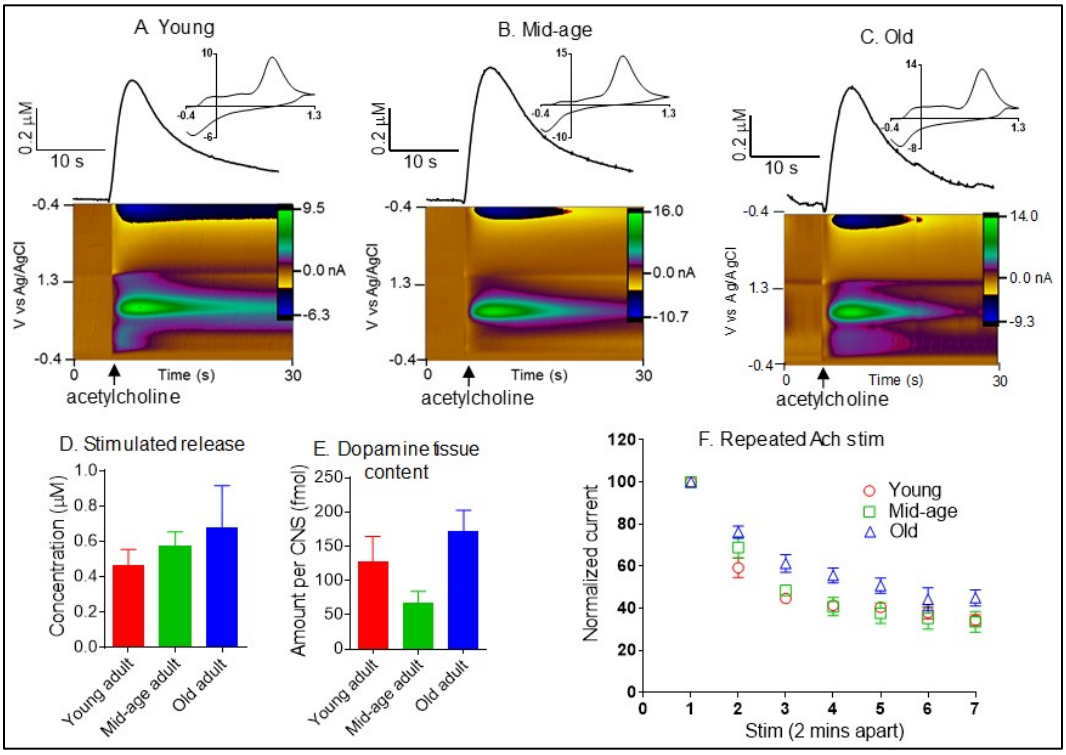

Figure 3.3. Acetylcholine-stimulated dopamine release in *Drosophila melanogaster* brain and tissue content in the CNS. Example plots for stimulated release in (A) young (1-5 day), (B) mid-age (20-25 day), and (C) old age adults (40-45 day). (D) Average concentrations of evoked dopamine for the different age groups. Although there is a slight increase in evoked dopamine with age, the difference is not significant (one-way ANOVA, $p = 0.6474$, n = 4-6). (E) The tissue content of dopamine in *Drosophila melanogaster* CNS at different ages. There was no significant effect of age on the tissue content of aging flies (one-way ANOVA, $p = 0.0758$, n = 5-8). (F) Acetylcholine stimulation was repeated at 2 min intervals and the responses were normalized to the first evoked current. With repeated stimulations, there are main effects of both age and stimulation number on evoked dopamine release but no interaction (2-way ANOVA, $p < 0.05$ for age and $p < 0.0001$ for stimulation number, interaction $p = 0.1314$).

To see if there are any changes in dopamine dynamics with age, acetylcholine-stimulated dopamine release was measured at 2 min inter-stimulation times. Newly synthesized dopamine and dopamine reuptake through transporters contribute to the releasable pool of dopamine.[35] It takes longer than 2 minutes for dopamine releasable pool to replenish in *Drosophila* larva.[36] On a short time scale, majority the releasable pool is maintained by reuptake, not synthesis.[36] Therefore the effect of reuptake on dopamine release with multiple

stimulations was studied by stimulating every two minutes. To correct for sample variability, the data were normalized to the peak concentration of the first stimulation (Fig. 3.3F). With repeated stimulations, there was a significant effect of age and a significant effect of stimulation number on evoked dopamine but no significant interaction (two-way ANOVA, $p < 0.05$, n = 4-6). Evoked dopamine is significantly lower with repeated stimulations, and response for old adult does not decrease as fast as it does for the mid-age adults. Surprisingly, the older flies are better able to maintain their dopamine release over multiple stimulations.

3.3.3 Stimulated dopamine and dopamine tissue content with age in Parkin knockdown

As mentioned above, there is a significant loss of dopaminergic cells during aging in humans. In patients with Parkinson's disease, the loss of dopaminergic cells in the substantial nigra is significantly higher than the control population.[37] We studied changes in evoked dopamine release with age in *Drosophila* with a knockdown of the *Parkin* gene with RNAi. Figure 3.4 A-C show example traces of concentration vs. time plot and CV at different ages for *Parkin-RNAi.* Previous work in our lab showed no difference in evoked dopamine release in *Parkin* knockout *Drosophila* larvae compared to control (data not shown), but since the disease is age related, we studied effects of *Parkin* knockout in adult *Drosophila* as they age (Fig. 3.4D).

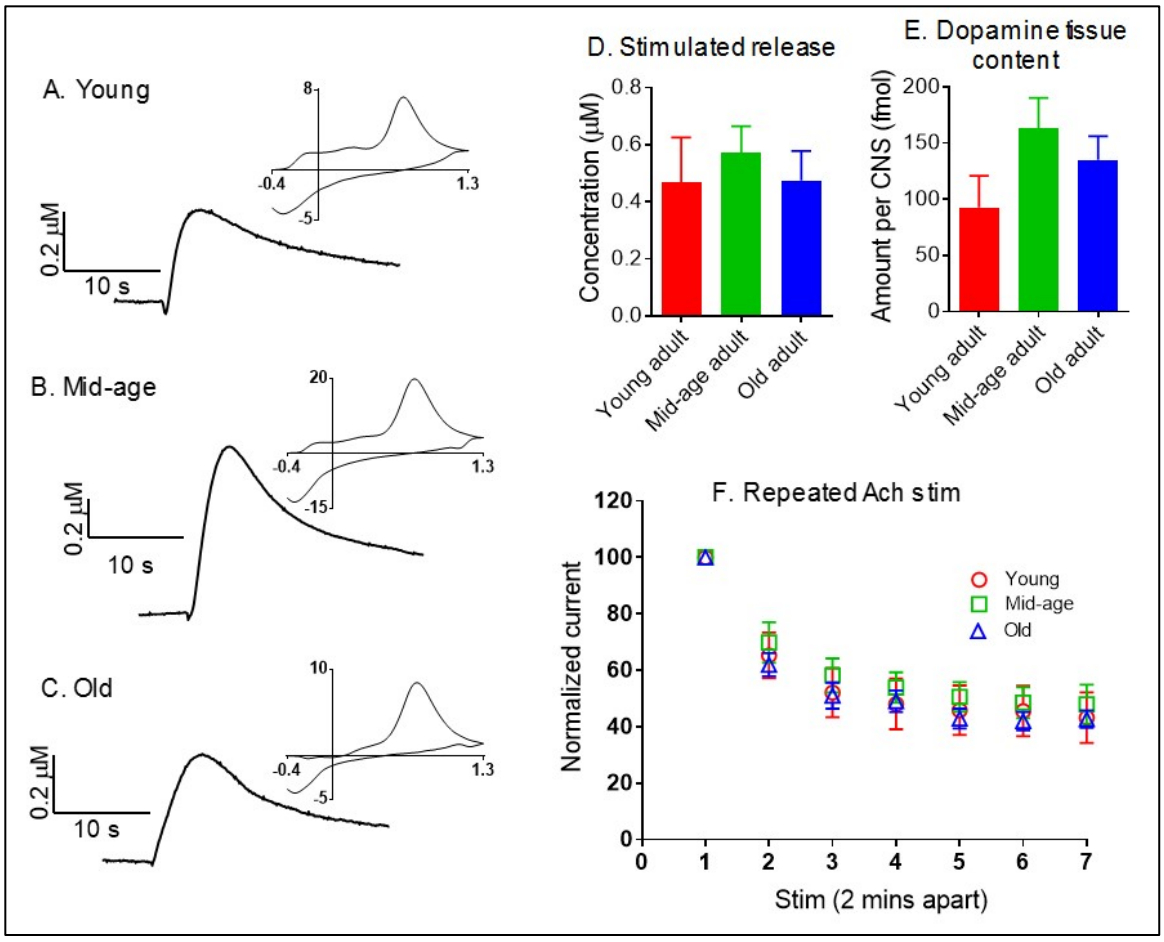

Figure 3.4. Acetylcholine-stimulated dopamine release in *Drosophila melanogaster* brain and tissue content in the CNS in *Parkin-RNAi.* Example plots for stimulated release in (A) young (1-5 day), (B) mid-age (20-25 day), and (C) old adults (40-45 day). (D) Average concentrations of evoked dopamine for the different age groups. There is no significant difference in stimulated release with age (one-way ANOVA, $p = 0.7977$, n = 5-6). (E) The tissue content of dopamine in *Parkin-RNAi Drosophila melanogaster* CNS at different ages. There was no significant effect of age on the tissue content of aging flies (one-way ANOVA, $p = 0.7543$, n = 6-7). (F) Acetylcholine stimulation was repeated at 2 min intervals and the responses were normalized to the first evoked current. With repeated stimulations, there was no significant effect of age, but a significant effect of stimulation number on evoked dopamine release (2-way ANOVA, $p = 0.7265$ for age and $p < 0.0001$ for stimulation number).

Acetylcholine stimulated release in the central complex of *Parkin* knockdown *Drosophila* was not significantly different at different ages (one-way ANOVA, $p = 0.7977$, n = 5-6). The tissue content of dopamine in the CNS of *Drosophila* was also not significantly different with age in the *Parkin* knockdown *Drosophila* (one-way ANOVA, $p = 0.7543$, n = 6-7) (Fig. 3.4E). Thus, the *Parkin*

flies were not more quickly depleted of dopamine. When acetylcholine stimulation was repeated every 2 minutes, and data were normalized, there was a significant effect of stimulation number (two-way ANOVA, $p < 0.0001$) but no effect of age (two-way ANOVA, $p = 0.7265$) (Fig. 3.4F). Thus, the *Parkin-RNAi* flies did not have a significant effect of age as the control flies did.

3.3.4 Stimulated dopamine and dopamine tissue content with age in RNF11 knockdown

Downregulating RNF11 has been proposed as a treatment for PD as we found that *RNF11* knockdown in *Drosophila* increases dopamine release and clearance in larval VNC.[31] However, we have not studied the effects of *RNF11* knockdown in adult *Drosophila*. Since Parkinson is an age related disease, we studied stimulated dopamine release in the central complex region of the brain of aging adult *Drosophila* with *RNF11* knockdown. Figure 3.5A-C show example traces of the release at different ages in *RNF11-RNAi* flies. There was no significant effect of age on stimulated dopamine concentration in *RNF11* knockdown *Drosophila* (one-way ANOVA, $p = 0.4422$, n = 5-6) (Fig. 3.5D). There was, however, a significant effect of age on the tissue content of dopamine in the CNS of aging *RNF11* knockdown *Drosophila* (one-way ANOVA, $p = 0.0284$, n = 5-6) (Fig. 3.5E). Old flies had more dopamine with the *RNF11* mutation.

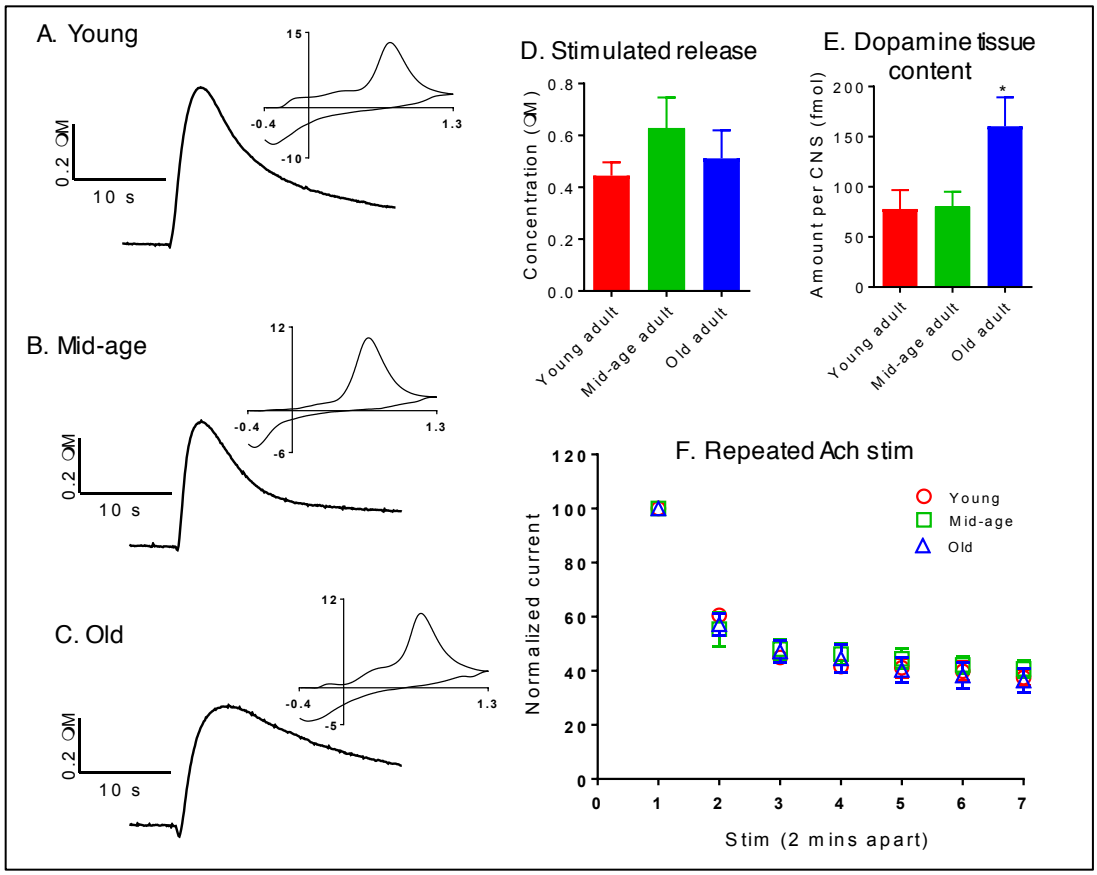

Figure 3.5. Acetylcholine-stimulated dopamine release in *Drosophila melanogaster* brain and tissue content in the CNS in *RNF11-RNAi.* Example plots for stimulated release in (A) young (1-5 day), (B) mid-age (20-25 day), and (C) old adults (40-45 day). (D) Average concentrations of evoked dopamine for the different age groups. There is no significant difference in stimulated release with age (one-way ANOVA, $p = 0.4422$, n = 5-6). (E) The tissue content of dopamine in *Parkin-RNAi Drosophila melanogaster* CNS at different ages. There was a significant effect of age on the tissue content of aging flies (one-way ANOVA, $p = 0.0284$, n = 5-6). (F) Acetylcholine stimulation was repeated at 2 min intervals and the responses were normalized to the first evoked current. With repeated stimulations, there was no significant effect of age, but a significant effect of stimulation number on evoked dopamine release (2-way ANOVA, $p = 0.8962$ for age and $p < 0.0001$ for stimulation number).

Evoked dopamine current was studied with repeated stimulations at 2 minutes inter-stimulation times at different ages. The current was normalized to the first dopamine oxidation peak current (Fig. 3.5F). Two-way ANOVA showed significant effect of stimulation number (two-way ANOVA, $p < 0.0001$, n = 5-6) but no effect of age on the release (two-way ANOVA, $p = 0.8962$, n = 5-6).

Similar to *Parkin-RNAi*, there was no significant effect of age observed in *RNF11-RNAi* with repeated stimulations. Therefore, only the control flies show a significantly higher release at old age when the stimulations were repeated at short time intervals and this effect is lost in the mutant flies.

3.3.5 Comparison of release with repeated stimulations in different lines

The 2-minute interval repeated stimulations data were compared among the different lines for the different age groups (Fig. 3.6). For all age groups, there was a significant effect of stimulation number but no significant effect of the genotype (two-way ANOVA, $p < 0.0001$ for stimulation number and $p > 0.1$ for genotype). For old-age control adults (Fig. 3.6A), the initial drop in stimulated current is not as great as for *Parkin* and *RNF11* knockdowns (Figs. 3.6B and C), but reaches similar levels on further stimulations. When only the first three stimulations are compared, there is a significant effect of genotype as the evoked current in the control line does not decay as much as in other lines (two-way ANOVA, $p < 0.05$). Therefore, for old-age control there is a significantly less decay in evoked current when compared to mid-age group, and also a significantly less decay in the three initial stimulations when compared to other genotypes.

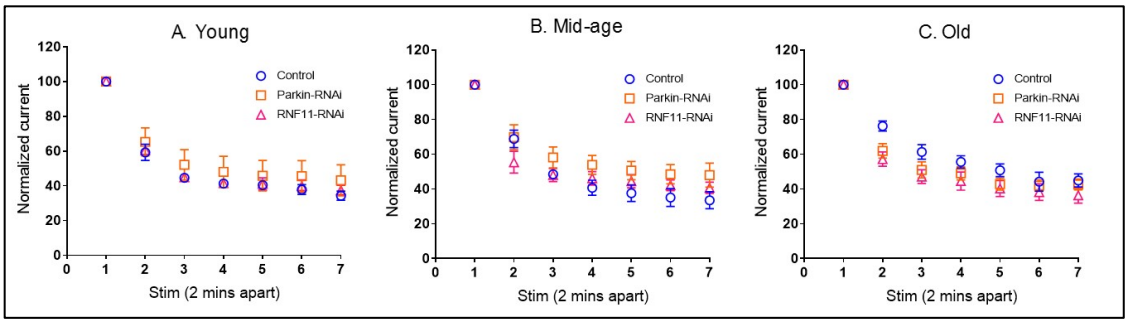

Figure 3.6. Acetylcholine-stimulated release in control, *Parkin-RNAi*, and *RNF11-RNAi Drosophila* repeated every 2 minutes and normalized to the first stimulation peak current. (A) Effect of genotype on young (1-5 day) flies. There was no significant effect of genotype, but a significant effect of stimulation number (2-way ANOVA, $p = 0.6438$ for genotype and $p < 0.0001$ for stimulation number). (B) Effect of genotype on mid-age (20-25 day) flies. There was again no significant effect of genotype, but a significant effect of stimulation number (2-way ANOVA, $p = 0.2216$ for genotype and $p < 0.0001$ for stimulation number). (C) Effect of genotype on old (40-45 day) flies. There was no significant effect of genotype, but a significant effect of stimulation number (2-way ANOVA, $p = 0.1352$ for genotype and $p < 0.0001$ for stimulation number).

3.4. Discussion

Parkinson's disease is a neurodegenerative disorder associated with aging that is characterized by a substantial loss of dopaminergic cells in the substantia nigra. *Drosophila melanogaster* is a popular model organism to study the disease, and there are many lines that model Parkinson's disease. Here, we studied effect of aging on stimulated dopamine release and tissue content in control flies, and flies with *Parkin* or *RNF11* knockdown. We found no significant change in the tissue content of dopamine in the CNS of *Drosophila* as they age normally or with *Parkin* knockdown, but there is a significant increase in the tissue content in old *RNF11-RNAi* flies. We found no significant effect of age on dopamine release with acetylcholine stimulation, but when stimulations are repeated at 2 min intervals there was a significant effect of age in control flies, but not in the knockdown lines. *Parkin* knockout mutation, therefore, does not

affect dopamine levels in the CNS, but affects dopamine dynamics in the central complex of the brain with age in *Drosophila* adults. When Parkinson's disease is diagnosed in humans, an approximately 80% of dopaminergic cells have already died. The higher levels of dopamine in *RNF11-RNAi* seen here suggests that the downregulation of RNF11 can be a compensatory mechanism to keep dopamine levels up in living cells when other cells degenerate. We show here that *Drosophila* can provide an understanding of fundamental changes in dopamine signaling that occurs with aging with Parkinson's disease.

3.4.1 Acetylcholine stimulated dopamine release in Drosophila melanogaster adult brain

Acetylcholine stimulated dopamine release in *Drosophila* larvae was recently discovered in our lab (Chapter 2). Here we measured stimulated dopamine in the central complex region of adult *Drosophila* brain. The central complex consists of five structures namely protocerebral bridge, fan-shaped body, ellipsoid body, paired noduli, and paired lateral accessory lobes.[38] The ellipsoid body and fan shaped body are the largest regions and thus it is most likely our electrode is implanted there. The central complex is rich in dopaminergic projections and the structure is involved in motor functions in *Drosophila*.[39-42] Also, the central complex and human basal ganglia, which contains the substantia nigra are considered evolutionarily conserved and share similar neurochemistry and behavioral outcomes.[38] The substantia nigra is the structure where a substantial loss of dopaminergic cells occurs in Parkinson's

disease.[37] The concentration of evoked dopamine in the central complex was dependent on the amount of acetylcholine stimulation with 10 pmol stimulation resulting in maximal response. Thus 10 pmol acetylcholine stimulation was used to study dopamine release in aging flies and in flies with Parkinson's disease mutations.

3.4.2 Stimulated dopamine release and dopamine tissue content during aging in Drosophila

The goal of this project was to study age dependent dopamine changes in *Drosophila* with Parkinson's disease mutations, and we first studied stimulated dopamine and dopamine tissue content in the CNS of control flies. Previous studies had shown that whole body dopamine levels of *Drosophila* decrease with age.[43] Another study in *Drosophila* also saw a decrease of TH labeling with age in the PPL2ab region of dopaminergic cells.[26] Here we observed no significant difference with age in either the concentration of acetylcholine-stimulated dopamine release or the tissue content of dopamine in the CNS of adult *Drosophila.* The result agree with other reports where GFP and antisera labeled TH had no significant decrease in *Drosophila* brains with age.[44] Although the whole body dopamine and subsets of dopaminergic cells decrease with age in *Drosophila,* our results and others show that there is no significant decrease of overall dopamine levels in the CNS or stimulated dopamine release in the central complex region. *Drosophila* exoskeleton contains dopamine so whole body measurements do not reflect the neurochemical functions. Our results show that

there is no deficit in dopamine function with age in the nervous system in normal *Drosophila*.

In humans there is a significant reduction of striatal TH activity and a decrease in the number of substantia nigra cells between the age of 20 to 90 years.[33, 34] The rate of dopaminergic cell death in the substantia nigra of the brain is 2.1 – 6.9% per decade in different parts of the substantia nigra in a control population.[37] There is also an age-related decline of dopamine transporters in various parts of the brain.[45] In *Drosophila*, there are reports of a decline of subsets of dopaminergic neurons, but our results show that it does not affect dopamine release in the central complex. Future studies should therefore investigate dopamine content and stimulated release in different regions of the *Drosophila* CNS to see if there is a region specific decline of dopamine content or release with age. We could also do staining of cells to verify the extent to which TH is decreased in our particular fly lines and the extent of dopaminergic cell death with age. With this information, we could better correlate cell death and neurochemistry in the future.

When acetylcholine-stimulation was repeated at 2 minutes inter-stimulation times, there was a significant effect of age and of stimulation number. The effect of stimulation number shows that dopamine releasable pool does not replenish when acetylcholine stimulation is repeated every 2 minutes. The result is similar to ATP stimulated release in transgenic *Drosophila* larvae expressing $P2X_2$ channels in dopaminergic neurons.[36] ATP-stimulated release went down to

about 60% in the transgenic larvae while acetylcholine-stimulated response went down to 34 – 45% in the adults on the 7th stimulation. The difference in decrease can be due to the difference in the stimulation method, life stage or region of the CNS tested. Future studies can therefore investigate tissue content in specific brain regions of adults and also study stimulated release in other regions of the adult brains to see if there are changes with aging. The significant effect of age on repeated stimulations with old-age having a less decrease compared to mid-age flies shows that the old flies maintain dopamine signaling better than younger flies when stimulations are repeated at 2 min intervals. Dopamine uptake is therefore maintained better in the old flies, and future research will investigate how uptake parameters change with aging flies.

3.4.3 Stimulated dopamine release and dopamine tissue content in Parkin knockdown Drosophila

Parkinson's disease model *Drosophila* show a significant loss of subsets of dopaminergic neurons and a decrease in TH expression.[14, 16, 46] We measured dopamine tissue content and stimulated release to see if *Parkin* knockout mutations have an effect on dopamine levels or tissue content. Since Parkinson's is an age related disease and expression of mutant human *Parkin* in *Drosophila* causes an age-dependent loss of dopamine neurons[45] we studied the effect of aging in dopamine signaling and content in *Parkin* knockout flies. There was no significant difference in either the CNS tissue content or stimulated concentration of dopamine in the central complex in aging *Parkin* knockdown flies. There was

also no significant difference of age when acetylcholine-stimulation was repeated at 2 minutes interval.

Compared to the control population, Parkinson's disease patients have a significantly higher dopaminergic cell loss of 45% in the substantia nigra during the first decade of a study.[37] The percent cell loss was different in different parts of the substantia nigra. We found that in *Parkin* knockdown *Drosophila,* there is no significant decrease in dopamine content in the CNS or in stimulated dopamine release as they age. Past studies have given conflicting evidence on the effect of *Parkin* knockdown on dopaminergic cells. Pesah et. al tested *Parkin* mutant *Drosophila* for dopaminergic cell loss in the dorsomedial clusters and found no significant difference in TH expression or any defect in the appearance of dopamine cell bodies in 3 week old *Parkin* mutant flies compared to control.[24] They did however observe a loss of flight muscles and suggested that *Parkin* may play an important role in cells with high energy demand. Another study using *Parkin* knockdown with RNAi also reported no difference in TH expression in posterior protocerebral dopaminergic neuronal clusters compared to control.[25] Yet another study found that there is a shrinkage of dopaminergic cells and reduced TH expression in dorsomedial dopaminergic cells of aged *Parkin* mutant flies compared to control, but other dopaminergic cells tested did not appear different in the mutant line.[32]

Therefore, it seems that *Parkin* mutants show variable effects on dopaminergic cells and there may be brain regions that are prone due to the

mutation while other regions that are not. Another possibility is that *Parkin* mutation alone is not enough for causing dopaminergic cell death. In humans, the disease is often linked to both genetic and environmental causes, and disease mutations do not always lead to Parkinson's disease.[47] Similarly in *Drosophila*, *Parkin* knockdown along with a toxin exposure caused dopaminergic cell death, but the mutation alone did not affect the cells.[25] It is therefore possible that some mutations in *Parkin* are more likely than others to cause the disease and that some mutations only increase the susceptibility to the disease. Two independent studies report that expression of mutant *Parkin* associated with human familial Parkinson's disease in *Drosophila* causes dopaminergic cell loss in neurons.[46] Future research should therefore examine *Drosophila* lines that have shown loss of dopaminergic cells and also focus on regions where cell loss has been observed.

3.4.4 Stimulated dopamine release and dopamine tissue content in RNF11 knockdown Drosophila

RNF11 is a negative regulator of the NF-kappa B pathway, and chronic activation of the pathway has been linked to neurodegeneration in Parkinson's disease. *RNF11* knockdown increased protection of dopaminergic cells *in vitro* and in rat 6-OHDA Parkinson's disease models.[30] The neuroprotection was suggested to be because of increased activity of NF-kappa B. We also found that *RNF11* knockdown in *Drosophila* increases dopamine release and clearance in larval VNC.[31] Here, we studied the effects of *RNF11* knockdown in aging adult

Drosophila. There was no significant difference in stimulated dopamine release with age but a significant difference in the tissue content with age. The old flies with *RNF11* knockdown had significantly higher dopamine levels in the CNS than younger flies – the effect that was not observed in either control or *Parkin-RNAi* flies. Therefore, *RNF11* knockdown acts to keep a high level of dopamine in the CNS. It is possible that the downregulation of *RNF11* in surviving cells of Parkinson's disease patients may be why symptoms only appear after up to 80% of cells have died as the total content of dopamine is maintained for a long time.

The stimulated dopamine result in adults is different than larvae, where *RNF11-RNAi* does have an effect on evoked concentration and the mutant line has a significantly higher evoked concentration than control.[31] It is important to note than the stimulations used in these studies are different. The stimulation used in this study (10 pmol acetylcholine) gives maximal response in control adults, but the stimulation used in larvae (2 s light stim: 60 Hz, 120 pulses, 4 ms red light each pulse) does not give maximal response in control adults. It is therefore possible that we are overstimulating the cells in adults, which is preventing us to observe any increase in dopamine release in the *RNF11-RNAi* line. Future studies should therefore use a smaller stimulation in both control and *RNF11-RNAi* lines to see if there will be any significant difference in evoked current response in the two lines. When acetylcholine-stimulations were repeated at 2 min intervals in *RNF11-RNAi* adults, there was an effect of stimulation number, but not of age. This is different than in controls where there was a

significantly less decrease at old age. *RNF11-RNAi* knockdown therefore affects the levels of dopamine with age and also how dopamine pool restores when there is a short interval between stimulations.

3.4.5 Repeated stimulations compared in different Drosophila lines

When the data for control vs. Parkinson's disease model flies were compared for different age groups, there was a significant effect of genotype when only the first three stimulations were compared. The old-age control group had a significantly lower decay during the first three stimulations than the *Parkin* or *RNF11* knockdowns. When all seven stimulations were compared, however, there was no effect of genotype on the old-age group. For young and mid-age groups, there was no significant effect of age on evoked current with repeated stimulations. The results show that with normal aging, the dopamine signaling is maintained better than aging with Parkinson's disease mutations.

3.5. Conclusions

We showed here that dopamine tissue content and stimulated dopamine release do not decrease with age in *Drosophila.* The tissue content is slightly but not significantly higher in older adult controls, and significantly higher in old age *RNF11-RNAi* adults. When acetylcholine-stimulations were repeated at 2 min intervals, there was a significant effect of age on control flies but not in the *Parkin-RNAi* or *RNF11-RNAi.* In the control flies there was less decrease of stimulated release for old flies, but the effect was not observed in *Parkin* or

RNF11 knockdowns. This suggests that as control flies age they have a faster replenishment of releasable dopamine pool compared to the Parkinson's disease mutants. No significant differences in dopamine content or release with aging in *Parkin* mutants suggests that the overall CNS content of dopamine is not affected in these mutants.

3.6. Future Studies

Drosophila melanogaster lines used to model Parkinson's disease in this study had a knockdown of *Parkin* or *RNF11-RNAi* genes in the dopaminergic cells. Knockdown with RNAi typically decreases the expression by about 50%, but is not a full knockout. Past research on *Parkin* knockouts provides evidence that not all dopaminergic cells are affected in these flies during aging.[24, 25, 32] Thus, it is important in the future to study additional brain regions as well. Models of the disease where mutant forms of human *Parkin* genes are expressed in *Drosophila* show degeneration of dopaminergic cells, and may be a better model to study the disease in the future.[4548] Future studies should also focus on the PPL and PAM regions, where dopaminergic cell degeneration has been observed in some *Parkin* mutants.[26, 48]

3.7. Experimental Section

3.7.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and solutions were prepared in Milli Q water (Millipore, Billerica, MA) unless noted

otherwise. Electrode calibrations were performed in phosphate buffer solution (PBS; 131.25 mM NaCl, 3.0 mM KCl, 10.0 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.0 mM Na_2SO_4, and 1.2 mM $CaCl_2$) with pH adjusted to 7.4, which was made once a month and stored at 4 °C. To make the dissection buffer, 11.1 mM glucose and 5.3 mM trehalose were added to the PBS buffer on the day of the experiment. A 10 mM stock solution of dopamine was prepared in 0.1 M $HClO_4$ once a month and stored at 4 °C. Dilute dopamine solution for electrode calibration was prepared in PBS buffer the day of the experiment. Acetylcholine chloride (5 mM) solution was prepared in PBS.

3.7.2 Drosophila dissection

Drosophila melanogaster strains were obtained from Subhabrata Sanyal at Biogen, Inc.: GFP expression driven by GAL4 on dopaminergic neurons (*TH-GAL4; UAS-GFP*); UAS-RNAi on Parkin (*UAS-Park-RNAi)*, and UAS-RNAi on RNF11 (*UAS-RNF11-RNAi*). Controls were *TH-GAL4; UAS-GFP. Drosophila melanogaster* stocks were maintained and crossed as described before.[49] For stimulated release experiments, the adult brains were dissected out following procedures described before, but here *Drosophila* were anesthetized on ice instead of carbon dioxide.[50] The dissected brain was transferred and plated to a petri dish with 3 mL dissection buffer.

For tissue content measurements, the whole CNS were dissected out in the dissection buffer following previously described methods with some

modifications.[51] The fly is held with tweezers on the abdomen and placed on a Sylgard-lined dish with buffer in it. A metal wire is used to pin the fly in the Sylgard through the abdomen. Next, the wings and legs are removed, and holding between the compound eye and the proboscis, the proboscis is also pulled out. The large trachea within the head capsule is removed. Next, the ventral part of the thorax is torn on both sides and the cuticle is removed. Tissues surrounding the VNC are cleaned out, and the VNC is carefully pulled while keeping it attached to the head. Next, grasping on the posterior corner of the head capsule with a set of tweezers, the compound eye is pulled out. The rest of the head capsule, compound eye and trachea are also removed taking care not the damage the brain to isolate the CNS.

3.7.3 Drosophila homogenate preparation

The tip of a gel-loading pipet is rotated on a flame to seal the tip. A fly CNS is transferred to the pipet tip with minimal dissection buffer. 2 µL of 2 mM perchloric acid is added to the sealed pipet tip. Next, a silver wire is used to break up the CNS and the sample vial is placed on a bath sonicator for 10 minutes. The sample vial is centrifuged at 14 rcf for 3 minutes. The sample vial is then inverted over a centrifugal filter and the sample is centrifuged again for 3 minutes. The filtrate is transferred to a 500 µL microcentrifuge tube.

3.7.4 Instrumentation and data analysis

Stimulated dopamine release measurements

Carbon-fiber microelectrode (CFME) was fabricated by previously described methods.[52] Briefly, a 7 μm diameter T-650 carbon fiber (Cytec Engineering Materials, West Patterson, NJ) were pulled into a 1.2 mm o.d. glass capillary. The capillary was pulled with a vertical puller to make two electrodes. The electrode was trimmed so 50 μm of carbon fiber was protruding. The tip of the electrode was epoxied to make a seal between the fiber and glass. The electrodes were calibrated with 1 μM dopamine in a flow cell.

Drosophila brain was viewed under a stereomicroscope (Nikon Instruments Inc., Melville, NY) and electrode was inserted into the central complex. The potential was applied between the CFME and a Ag/AgCl reference electrode in the bath. A picospritizing pipet was fabricated by pulling a 1.2 mm o.d. glass capillary, coating the tip with black Sharpie permanent marker for visualization of the tip. The pipet was trimmed and filled with acetylcholine. It was calibrated by measuring the diameter of liquid puffed on in oil at a set pressure and time. The pipet was also inserted into the brain about 10 μm away from the CFME.

Dopamine tissue content measurements

The working electrode used for tissue content measurements with capillary electrophoresis was carbon-fiber disk electrode fabricated with 30 μm diameter fiber (World Precision Instruments, Sarasota, FL). The CE with end column FSCV was built in house as described previously.[53] Electrokinetic

injection of the sample was performed by placing the end of the separation capillary into the microcentrifuge tube. Sample was injected by applying applying +5 kV for 15 secs . Separation of dopamine from other analytes was performed by applying +9 kV at the injection end of the capillary. The electrode was precalibrated with 100 nM dopamine.

Data were collected with Waveneuro potentiostat (Pine Research Instrumentation, Durham, NC). HDCV software (a gift of Mark Wightman, University of North Carolina) was used for data collection and analysis. All statistics were performed in GraphPad Prism 6 (La Jolla, CA). Significance was measured with a 95% confidence interval. Graphs are shown as mean ± standard error of the mean.

3.8. References

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Chapter 4: Fast-scan cyclic voltammetry (FSCV) detection of endogenous octopamine in *Drosophila melanogaster* ventral nerve cord

Chapter 4: Fast-scan cyclic voltammetry (FSCV) detection of endogenous octopamine in *Drosophila melanogaster* ventral nerve cord

4.1. Abstract

Octopamine is an endogenous biogenic amine neurotransmitter, neurohormone, and neuromodulator in invertebrates, and has functional analogy with norepinephrine in vertebrates. Fast-scan cyclic voltammetry (FSCV) can detect rapid changes in neurotransmitters, but FSCV has not been optimized for octopamine detection *in situ.* The goal of this study was to characterize octopamine release in the ventral nerve cord of *Drosophila* larvae for the first time. An FSCV waveform was optimized so that the potential for octopamine oxidation would not be near the switching potential where interferences can occur. Endogenous octopamine release was stimulated by genetically inserting either the ATP sensitive channel, $P2X_2$, or the red-light sensitive channelrhodopsin, CsChrimson, into cells expressing tyrosine decarboxylase (TDC), an octopamine synthesis enzyme. To ensure that release is due to octopamine and not the precursor tyramine, the octopamine synthesis inhibitor disulfiram was applied, and the signal decreased by 80%. Stimulated release was vesicular and a 2 s continuous light stimulation of CsChrimson evoked 0.22 ± 0.03 μM of octopamine release in the larval VNC. Repeated stimulations were stable with 2 or 5 minutes interstimulation times. With pulsed stimulations, the release was dependent on the frequency of applied light pulse. An octopamine transporter has not been identified, and blockers of the dopamine transporter and serotonin transporter had no significant effect on the clearance time of

octopamine, suggesting they do not take up octopamine. This study shows that octopamine can be monitored in *Drosophila*, facilitating future studies of how octopamine release functions in the insect brain.

4.2. Introduction

Octopamine and its precursor, tyramine, are biogenic amines in invertebrates with distinct roles that range from modulation of muscles to complex social behaviors.[1, 2] Octopamine acts as a neurotransmitter by regulating intracellular cyclic AMP and Ca^{2+} in *Drosophila melanogaster*.[3] In larvae, octopamine/tyramine neurons in the ventral nerve cord (VNC) control locomotion, and in adults, they control locomotion, sensitivity to cocaine, and egg laying activity.[4, 5] Octopamine also has many neuromodulatory functions including regulating neuromuscular transmission in larvae, and modulating learning, memory, and conditional courtship in adults.[6-8] When released into the hemolymph, it acts as a neurohormone to mobilize lipids and carbohydrates during energy-demanding processes.[9, 10] Octopamine is found in trace amounts in vertebrates and as a trace amine may play a role in depression and other psychiatric disorders.[11-13] Octopamine in invertebrates is also analogous in function to norepinephrine in vertebrates. For example, octopamine release can be stimulated by nicotine application in *Drosophila,* similar to norepinephrine release in mammals.[14]

Octopamine, a phenol, is an electroactive compound, and can be detected via direct electrochemistry.[15] The mechanism involves a one-electron oxidation at the phenol group that creates a radical. The radical can react with other octopamine molecules to create an electroactive polymer, and a secondary oxidation peak due to oxidation of this polymer is also observed.[16] Octopamine has been detected in biological samples after separation by coupling high performance liquid chromatography or capillary electrophoresis to electrochemical detection.[17-19] Separation methods are useful in obtaining tissue content information, but are destructive and unable to provide information on real-time dynamics of octopamine.

The main electrochemical methods for studying real-time neurotransmitter release *in vivo* are amperometry, chronoamperometry, and fast-scan cyclic voltammogram (FSCV). Majdi et al. recently measured endogenous octopamine release using optogenetic stimulation in a *Drosophila* larval neuromuscular junction preparation.[20] They used amperometry at 0.9 V, and estimated the number of molecules of octopamine released per vesicle. Amperometry is sensitive and has high temporal resolution, but the technique is not selective and is more difficult to use in a complex preparation. Chronoamperometry measures a ratio of oxidation to reduction currents at given potentials. Fuenzalida-Uribe et al. used chronoamperometry to detect octopamine evoked by nicotine stimulation of α-bungarotoxin-sensitive nAChRs in adult *Drosophila* brains.[14] With fast-scan cyclic voltammetry (FSCV), the voltage is ramped up and back to oxidize and

then reduce the molecule of interest. While a background current must be subtracted, the resulting background-subtracted cyclic voltammogram (CV) helps identify the compound detected. An FSCV waveform has been optimized for stable *in vitro* analysis of octopamine at carbon fiber microelectrodes (CFMEs).[16] Real-time measurements of dopamine and serotonin have been made in *Drosophila* ventral nerve cords (VNC) using FSCV but the method has not been extended to studying octopamine dynamics.[21, 22]

Here, we optimize an FSCV waveform and detect stimulated octopamine release in the *Drosophila* larval ventral nerve cord (VNC) for the first time. By using a red-light activated ion channel, CsChrimson, there is no background shift close to the octopamine oxidation peak due to the photoelectric effect.[23, 24] Octopamine release was also verified by picospritzing octopamine into the tissue.[25] CsChrimson or $P2X_2$, an ATP activated channel, were expressed in neurons expressing the tyrosine decarboxylase (*Tdc2*), the enzyme used to synthesize the octopamine precursor, tyramine. Since these cells have both tyramine and octopamine, disulfiram, an inhibitor of tyramine *β*-hydroxylase (TBH), the enzyme that converts tyramine to octopamine, was used to demonstrate that the majority of the release is octopamine.[26-28] Octopamine release was vesicular and not cleared by the dopamine transporter (DAT) or serotonin transporter (SERT). These are the first measurements of endogenous octopamine release in the *Drosophila* larval central nervous system, which will

facilitate studies of the function of octopamine signaling in an intact nervous system.

4.3. Results and discussion

4.3.1 Visualization of octopaminergic neurons

The UAS-GAL4 system allows targeted expression of certain proteins to specific cells or tissues.[29] In this study, the *GAL4* gene was expressed in cells expressing *Tdc2*, neuronal tyrosine decarboxylase (TDC).[30] Tyrosine decarboxylase converts tyrosine to tyramine, which is then converted to octopamine by tyramine *β*-hydroxylase (TBH). The transcriptional activator, GAL4, activates UAS (Upstream Activation Sequence) and transcribes genes that are under its control – in this case genes coding for GFP, CsChrimson, or $P2X_2$. Thus, those genes are expressed only in neuronal cells expressing TDC.

To characterize the location of octopaminergic projections, the *UAS-GFP* reporter gene was expressed under the control of *Tdc2-GAL4* driver. The cell bodies, which are unpaired median neurons, are located primarily in the middle of the abdominal section of the VNC (marked a1 – a9 in Fig 4.1A) and show strong GFP expression.[31] The projections extend from the middle out to the side through the neuropil. The expression pattern is similar to previously observed patterns except that here, GFP was not expressed as strongly in the thoracic section (t1 – t3, Fig 4.1A).[4] The electrode was therefore placed in the abdominal segment 2-4 in the neuropil, where high levels of octopaminergic projections are present.

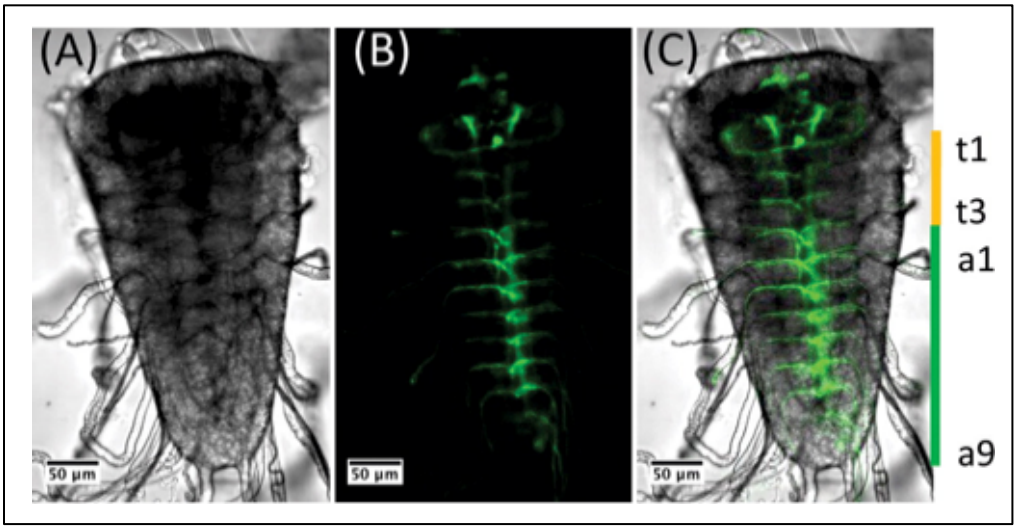

Figure 4.1. Visualization of neurons expressing tyrosine decarboxylase (Tdc) in *Drosophila melanogaster* larval ventral nerve cord (VNC). (A) Bright field image of a larval VNC and (B) image of the same VNC expressing GFP in neurons with tyrosine decarboxylase (Tdc2–GAL4; UAS–GFP). (C) Overlay of GFP expression on the image of the VNC. Abdominal section (a1–a9) and thoracic section (t1–t3) are indicated on the right. Strong GFP expression (green) is seen on the unpaired median neurons in the abdominal section, with projections into the neuropil on either side

4.3.2 Optimization of FSCV waveform for octopamine detection

FSCV can be used to detect octopamine but an optimized waveform for *in vivo* use had not been developed. In this first study, we characterized octopamine detection with different waveforms *in situ* to optimize octopamine detection. First, a positive waveform, that held at a positive voltage, 0.1 V, scanned up to 1.3 V and back at 600 V/s was tested, because it had been optimized previously *in vitro*.[16] This waveform was termed the positive waveform because of the positive holding potential. Figure 4.2A shows CsChrimson stimulated octopamine *in situ* (2 s stimulation) with the applied positive triangular waveform (top), cyclic voltammogram profile (middle), and color plot (bottom). The CV for the positive waveform has a peak around 1.2 V and the color plot shows that the oxidation peak is on the cathodic, downward scan very close to the switching potential. The

peak potential is more shifted than for *in vitro* measurements, where the primary peak is observed around 0.9 V at this waveform,[16] because the tissue can foul the electrode and slow electron transfer, changing the shape of the cyclic voltammogram.[32] The positive waveform CV has very little secondary peak, because the oxidation products of octopamine are positively charged and are repelled by the positive holding potential.

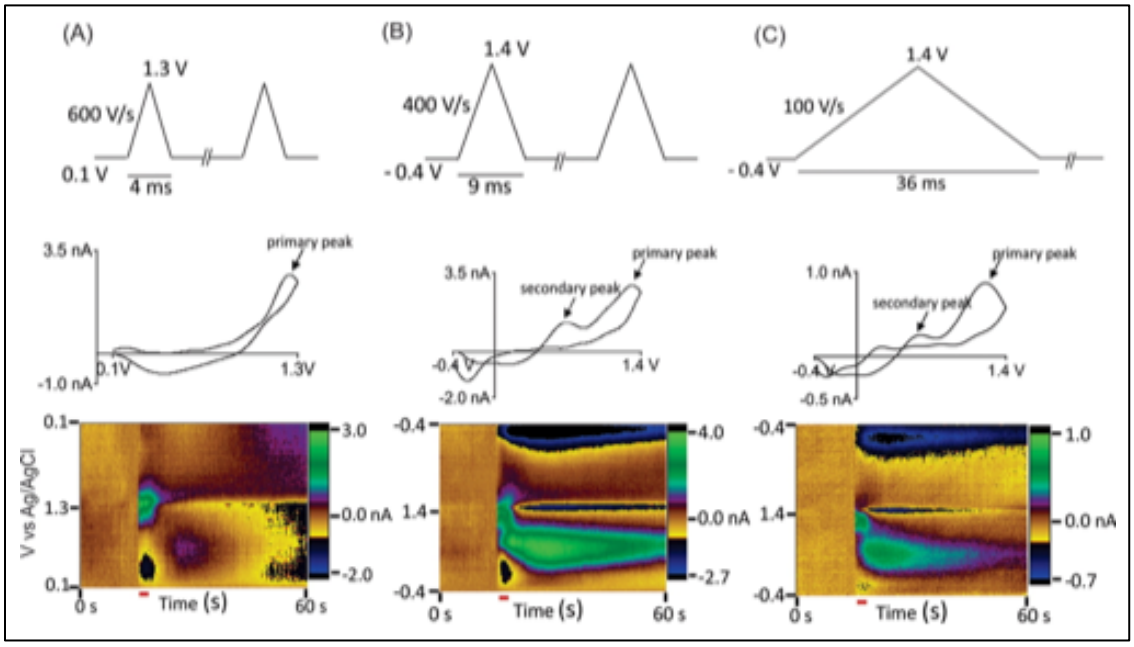

Figure 4.2. Optimization of FSCV waveform for octopamine detection. The applied waveform (top), cyclic voltammogram (center), and color plot (bottom) for octopamine detected with (A) positive waveform, (B) dopamine waveform, and (C) slower scan waveform. The 2 s stimulation is marked by a red line under the color plot.

Next, we tested a waveform commonly used for dopamine detection, scanning from -0.4 to 1.4 V and back at 400 V/s. The primary peak for octopamine is observed around 1.3 V, close to switching potential *in situ* (Fig. 4.2B). The secondary peak is around 0.7 V and the color plot shows it lasts a long time, which suggests the oxidation products of octopamine are sticking to

the electrode. When repeated calibration measurements are made *in vitro*, the response for octopamine decreases with the dopamine waveform due to fouling of the electrode by the secondary products.[16]

In order to move the octopamine oxidation peak away from the switching potential, we optimized a waveform using a slower scan rate than the dopamine waveform. Figure 4.2C shows that with a 100 V/s scan rate, the main peak for octopamine is at 1.1 V on the anodic scan *in situ*, away from the switching potential. The secondary peak is present at 0.56 V, which aids identification, but it is not as intense and does not last as long as for the dopamine waveform. Slower scan rates do give lower Faradaic currents, as current is proportional to scan rate. Decreasing the scan rate does decrease the sensitivity from an average of 12 ± 1 nA/μM for 400 V/s to 4.3 ± 0.5 nA/μM for 100 V/s. However, noise is also proportional to background current and decreases as well. The average S/N ratio for octopamine detection for 1 mM octopamine with the slower scan waveform is 154, which is 88% of the average S/N ratio of 174 for the dopamine waveform. The LOD calculated from the average S/N ratios of 200 nM octopamine for the dopamine waveform is 14 nM and for the slower scan waveform it is 19 nM.[33] The slower scan waveform was chosen for the rest of the studies because the background current is most unstable at the switching potential, and so errors (such as pressure changes with pressure ejection) often occur at this potential. Errors at the switching potential are a problem when using blue light sensitive Channelrhodopsin (ChR2) to mediate octopamine release

because the photoelectric effect causes small currents at the switching potential.[24] Thus, using the slower scan rate waveform keeps the main peak away from the switching potential and allows the secondary peak to be used as an identifier. While the secondary peak is present for this waveform, we show *vide infra* that stable current responses are observed for octopamine upon repeated stimulations.

4.3.3 Confirmation the released compound is octopamine and that release is vesicular

Since the CsChrimson is expressed in cells expressing the tyramine synthesis enzyme, TDC, they could be releasing octopamine or tyramine. Disulfiram, a tyramine *β*-hydroxylase (TBH) inhibitor, was applied to block the synthesis of octopamine from tyramine. Thus, a decrease in stimulated release after disulfiram would confirm that the release was octopamine.[26-28] Continuous, 2 s red light stimulations of CsChrimson were repeated every 5 min after bathing VNCs in 100 μM disulfiram for 15 min. Results were normalized to the value of the first stimulation. For control flies, release from repeated stimulations were consistent and the eighth stimulation (45 mins in buffer + DMSO) was 94 ± 3% of the first, which was not a significant decrease (One-way ANOVA, Bonferroni post-test, $p > 0.05$) (Fig. 4.3). Disulfiram significantly decreased evoked current (one-way ANOVA, $p < 0.0001$, n = 6), and the current decreased to 19 ± 3% after the eighth stimulation (45 mins in disulfiram) (Bonferroni post-test, $p < 0.05$). The magnitude of the decrease is similar to the decrease in stimulated serotonin after

the use of a serotonin synthesis inhibitor.[34] This substantial decrease in current after disulfiram indicates that the majority of the evoked species is octopamine. Future studies could also confirm this genetically by blocking targeted expression of the enzyme tyramine *β*-hydroxylase with RNAi. However, a stable homozygous line expressing *tdc2-GAL4; UAS-CsChrimson* is not currently available, so all three genes – *tdc2-GAL4, UAS-CsChrimson*, and *UAS-RNAi*$^{T\beta H}$ – could not be expressed together.

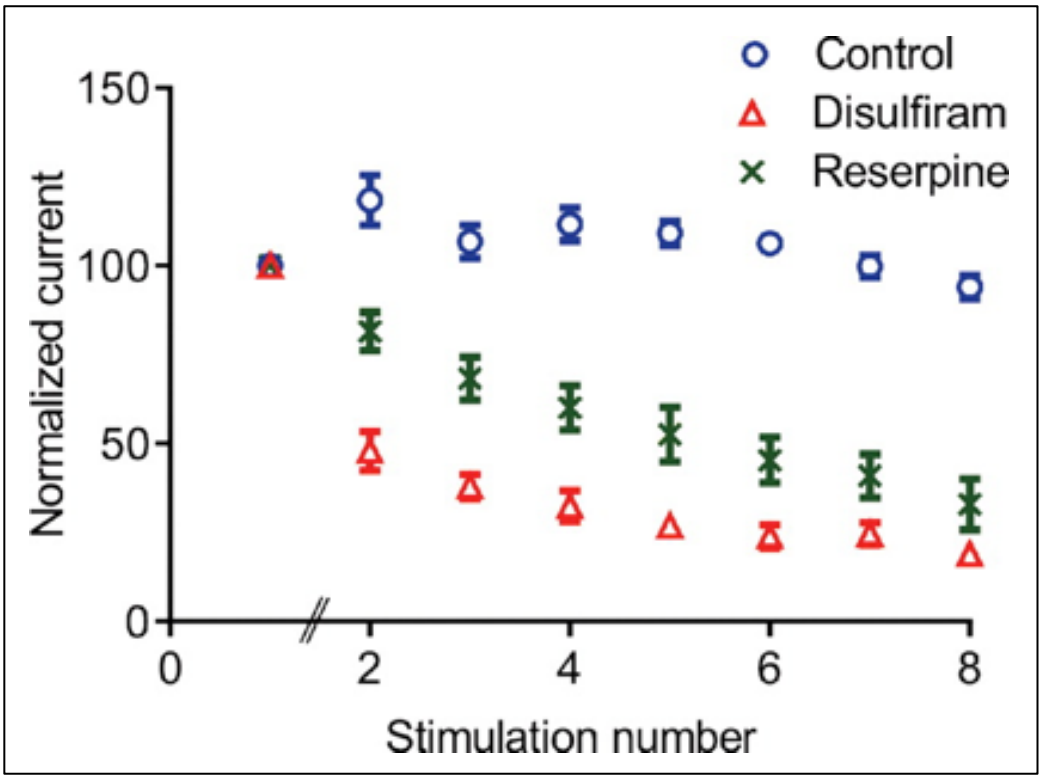

Figure 4.3. Confirmation of the detection of octopamine and that the release is vesicular. After the initial stimulation predrug (stimulation number 1), the VNCs were bathed in 100 μM disulfiram to inhibit octopamine synthesis or 100 μM reserpine to block the vesicular monoamine transporter. After 15 min in drug, stimulations were performed every 5 min after the second stimulation. The evoked current is significantly lower than control for both disulfiram and reserpine (one-way ANOVA, $p < 0.0001$, $n = 6$).

Neurotransmitters are contained in vesicles, which release their contents into the synaptic cleft by exocytosis after an action potential. To determine if

octopamine release is vesicular, VNCs were bathed in 100 µM reserpine, a vesicular monoamine transporter (VMAT) inhibitor. VMAT is a protein that transports neurotransmitters from the cytosol into synaptic vesicles for storage and subsequent release. There was a significant effect of reserpine on evoked current (one-way ANOVA, $p < 0.0001$, n = 6) and evoked current decreased 33 ± 7 % on the eighth stimulation (45 mins in reserpine) (Bonferroni post-test, $p < 0.05$ compared to control). Reserpine is likely not blocking 100% of VMAT or the releasable pool may not be depleted, so the response does not go to zero. The results confirm that optogenetic stimulation is causing vesicular release of octopamine. The vesicular nature of octopamine release could also be probed further with genetic manipulation of the VMAT gene in the future.[35]

4.3.4 Comparison of puffed on, CsChrimson, and P2X2-mediated octopamine release

The CVs and color plot profiles of stimulated release were compared to those obtained from puffing octopamine into VNCs. Octopamine (0.01 pmol) was applied in the VNC using a picospritzing pipette (Fig. 4.4A). Stimulated release was compared for optically activated CsChrimson channels and ATP activated $P2X_2$ channels. Figure 4.4B shows the CV (top right), concentration vs. time (center), and color plot (bottom) obtained for 2 s continuous red light stimulation of *Tdc2-GAL4; UAS-CsChrimson* larval VNC. On average, a 2 s stimulation of CsChrimson released 0.22 ± 0.03 µM octopamine. Figure 4.4C shows similar plots for ATP stimulation of *Tdc2-GAL4; UAS-$P2X_2$* larval VNC preparation. On

average, ATP stimulation released 0.33 ± 0.06 μM of octopamine. The higher level of release with ATP compared to 2 s red light stimulation is consistent with previous observations of stimulated dopamine release.[24, 36] The CVs for octopamine stimulated by CsChrimson and $P2X_2$ are similar to the CV for exogenously applied octopamine with primary peaks around 1.1 V and secondary peaks around 0.5 V. The peak close to 1.3 V on the CV for ATP stimulated release is an artifact (as marked in Figure 4.4C), which is likely due to background shifts from pressure ejection or ATP oxidation.[36] With a higher scan rate waveform, this artifact would interfere with the primary oxidation peak for octopamine, but at 100 V/s, the octopamine oxidation peak and the artifact are well separated and there is no interference. The concentration vs. time plot for CsChrimson stimulated release has a sharper peak than ATP stimulated release, because the timing is easier to control with light stimulations than with ATP, which must diffuse through the tissue and is present longer. This longer course of ATP stimulation also explains the higher levels of release and longer lasting secondary peaks obtained compared to optogenetic stimulation. In future experiments, either light or ATP-mediated release could be used to study octopamine dynamics.

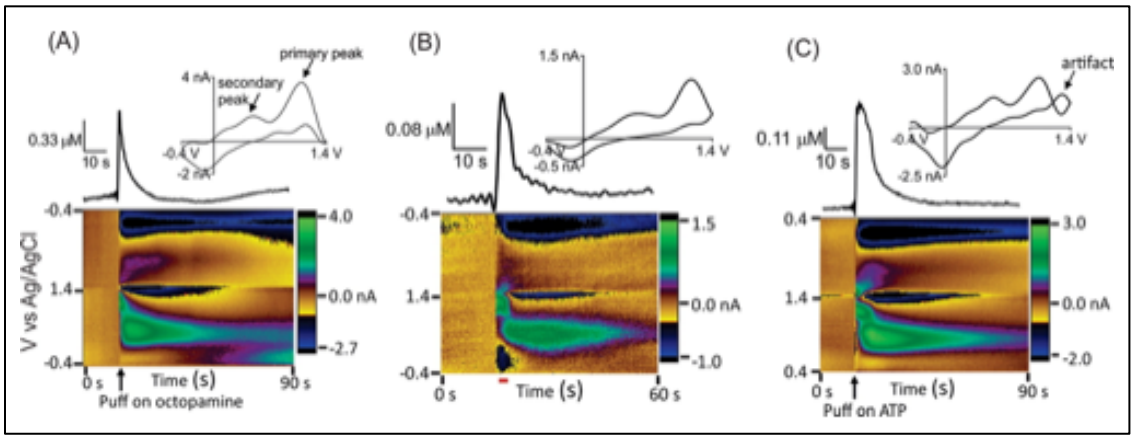

Figure 4.4. Comparison of puffed-on octopamine and different stimulations. Cyclic voltammograms (top), concentration vs time (center), and color plots (bottom) for (A) octopamine puffed into a larval VNC, (B) CsChrimson (red light, 2 s) mediated release, and (C) P2X2 (0.5 pmol of ATP) mediated release in larval VNC. All show similar peak characteristics with the primary peak around 1.1 V and secondary peak around 0.5 V with the slower scan waveform.

4.3.5 Stability of release and effect of stimulation frequency

Red light-stimulated release via CsChrimson was further characterized by studying the stability of octopamine release and the effect of interstimulation times on stability. Two second duration, continuous red light stimulations were repeated every 2 or 5 minutes. The release was stable with both 2 minute (one-way ANOVA, $p = 0.2651$, n = 7) and 5 minute (one-way ANOVA, $p = 0.8197$, n = 7) interstimulation times (Fig. 4.5A). Similarly, a stable current response was also obtained upon repeated exposures to octopamine at these intervals for *in vitro* calibrations (one-way ANOVA, $p > 0.5$, n = 4) (Fig. 4.5B). Stable release suggests that the secondary oxidation product does not foul the electrode and cause changes in sensitivity on this time scale. The standard error of the mean was higher and currents slightly lower for the 2 minute interstimulation time; hence a 5 minute interstimulation time was used for further experiments.

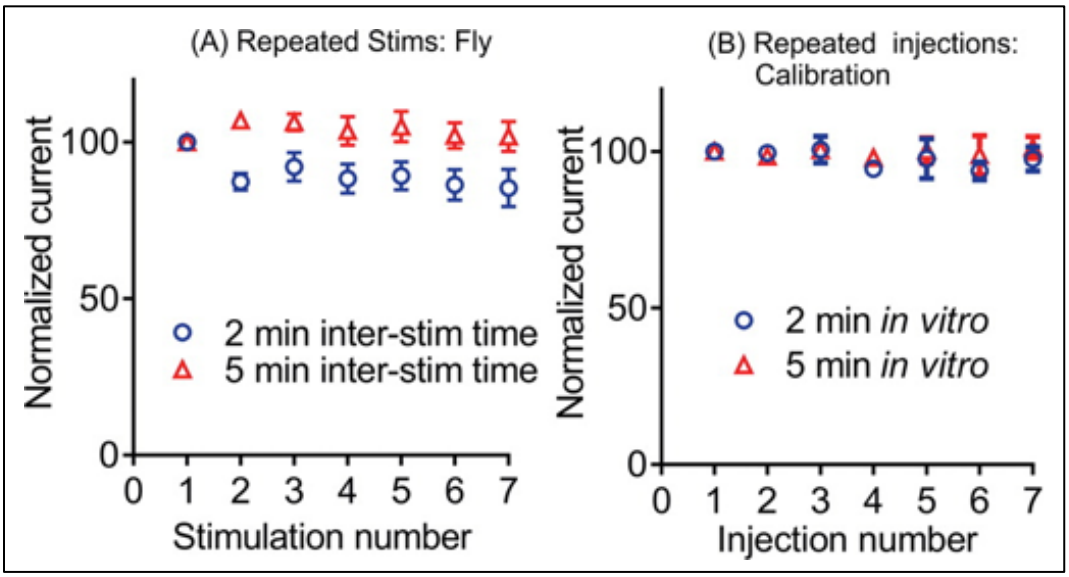

Figure 4.5. Stability of CsChrimson stimulated octopamine release. (A) The release evoked by 2 s red light stimulation is stable for 2 min interstimulation time (ANOVA, $p = 0.2651$, $n = 7$) and 5 min interstimulation time (ANOVA, $p = 0.8197$, $n = 7$). (B) Stable responses are also observed for *in vitro* calibrations for 2 s octopamine injections with 2 min (ANOVA $p = 0.7441$, $n = 4$) or 5 min ($p = 0.9920$, $n = 4$) interevent times.

Continuous red light stimulations were varied between 0.1 s and 3 s in order to test the effect of stimulation length on octopamine release (Fig. 4.6A). Octopamine oxidation current increased with increasing duration of light stimulation up to 0.5 s. With stimulations longer than 1 s, the evoked current decreased after reaching a maximum level, even while the light was still on. This decreasing response is different than the signals for dopamine and serotonin that keep increasing or reach steady state during long stimulations.[22, 34] The results suggest that the releasable pool for octopamine is more limited than for serotonin or dopamine in similar preparations.

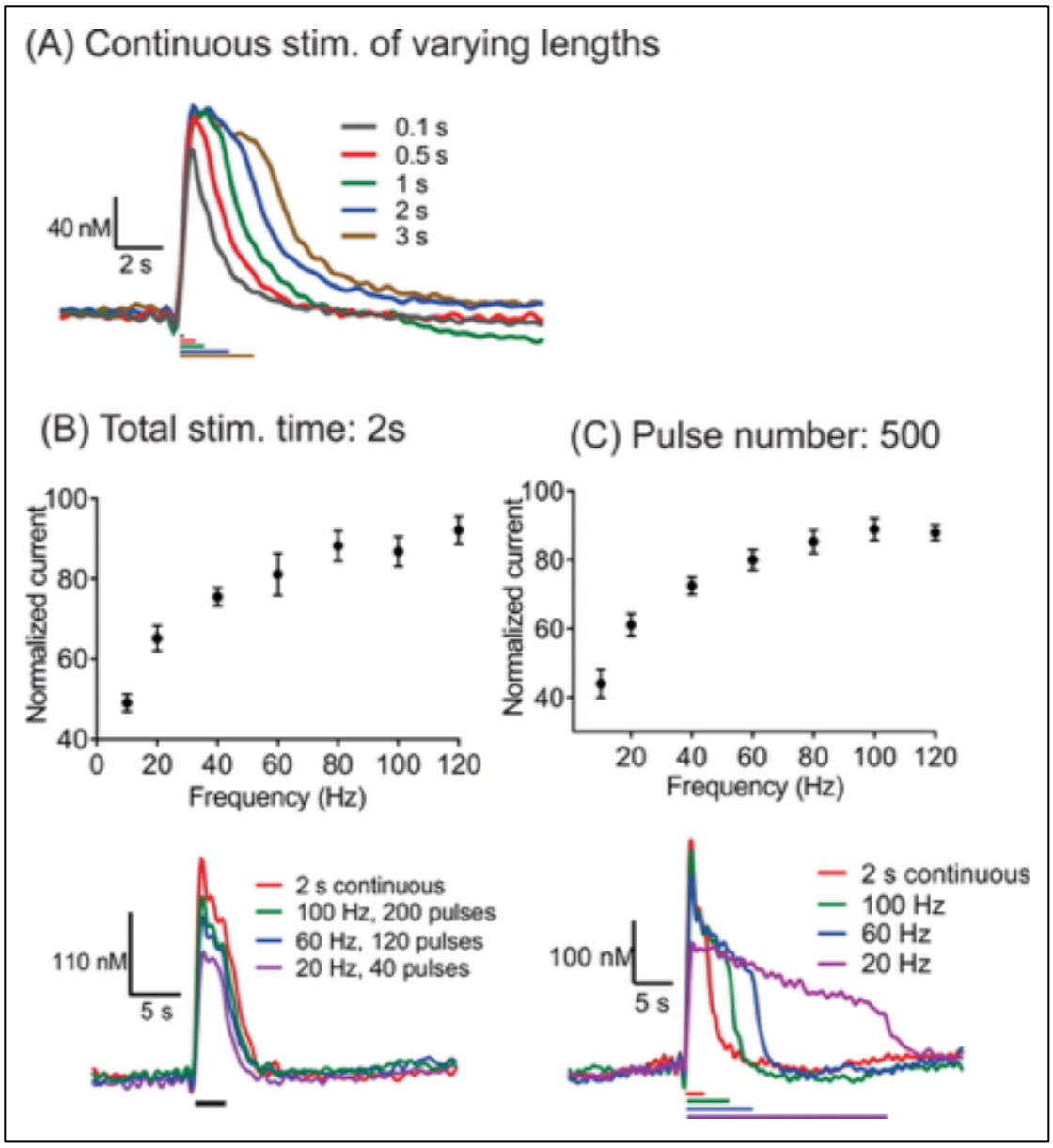

Figure 4.6. Effects of length of continuous light stimulation and frequency of pulsed stimulations. (A) Concentration vs. time plots for continuous light stimulation of different durations. Colored bars at bottom show total stimulation times. (B,C) Effect of pulsed stimulation frequency. (B) The total stimulation time was constant at 2 s and pulse length was 4 ms. Data are normalized to 2 s continuous stimulation set to 100%. Current due to evoked octopamine is dependent on the frequency of applied light and significantly different than the 2 s continuous stimulation up to 60 Hz (one-way ANOVA, Bonferonni post-test, $p < 0.0001$, $n = 7$). Example concentration vs. time plots are shown below with colored bars showing total stimulation time. (C) The total number of pulses was kept constant at 500 and length of pulse at 4 ms so the total time light is on is 2 s. There was a significant effect of frequency up to 80 Hz (Bonferroni's multiple comparisons test, $p < 0.05$).

Pulsed light stimulations are often used to mimic neuronal firing patters.[37, 38] To understand the effect of pulsed stimulation and changing frequency of stimulation, pulse trains were studied in two ways: (1) holding the stimulation time constant at 2 s (i.e. changing the frequency and number of pulses), and (2) holding the duration of the light on constant for 2 s (i.e keeping the number of pulses constant at 500 pulses while changing the frequency). All light pulses were 4 ms in length. The currents were normalized to a 2 s continuous stimulation for each sample. For the first stimulation method with constant stimulation time, current was dependent on the frequency of pulsed light (Fig. 4.6B, One-way ANOVA, $p < 0.0001$, n = 7), and was significantly different from the 2 s continuous stimulation up to 60 Hz (Bonferroni's post-test, $p < 0.05$). Figure 4.6B also shows example current vs. time traces that illustrate that higher concentrations of octopamine were released with higher frequencies. For the second stimulation method, with constant pulses, there was a significant main effect of frequency (Fig. 4.6C, one-way ANOVA, $p < 0.0001$, n = 7) and post-tests showed that signals were significantly different than the 2 s continuous stimulation up to 80 Hz (Bonferroni's post-test, $p < 0.05$). The concentration vs. time plots (Fig. 4.6C) show that the concentration of octopamine decreases with time as the length of stimulation increases, which is clearly seen in the 20 Hz plot. Similar to the continuous stimulations (Fig. 4.6A), the concentration of octopamine decreases during the stimulation, which implies that the releasable pool can be easily depleted. In contrast, for other neurotransmitters like

serotonin, a steady state response is observed at lower frequencies instead of a decrease.[38] Overall, results of the pulsed stimulation tests show the evoked current is dependent on the frequency of light; however the octopamine release is depleted with longer stimulations and decreases while the light stimulation is being applied.

4.3.6 Characterization of octopamine clearance

Most monoamine neurotransmitters are cleared back into cells via a transporter, but an octopamine transporter has not been identified in *Drosophila.*[39] Octopamine has a similar structure to dopamine, and it has been suggested that the dopamine transporter could transport octopamine.[40] However, tests of *Drosophila* dopamine transporter (dDAT) expressed in MDCK cells found that octopamine was not a good substrate for dDAT.[41] *Drosophila* serotonin transporter (dSERT) expressed in HeLa cells and *Xenopus oocytes* have also shown little or no activity for octopamine.[42, 43] However, the clearance of octopamine by known monoamine transporters has not been studied in the VNC. We tested the extent to which the dDAT or dSERT affect octopamine clearance by using nisoxetine to inhibit dDAT and fluoxetine to inhibit dSERT.[22, 44] Figure 4.7 shows example data before and after the uptake inhibitors. There is little effect of 20 mM nisoxetine, and 100 mM fluoxetine increases the peak height but not the clearance time. To quantify clearance, t_{50}, the time the concentration vs. time curve to decay from the peak to 50% was calculated and it was not significantly different for nisoxetine or fluoxetine compared to control values

(paired t test, $p > 0.1$, n = 5 - 8) (Table 1). Thus, dSERT or dDAT do not uptake octopamine.

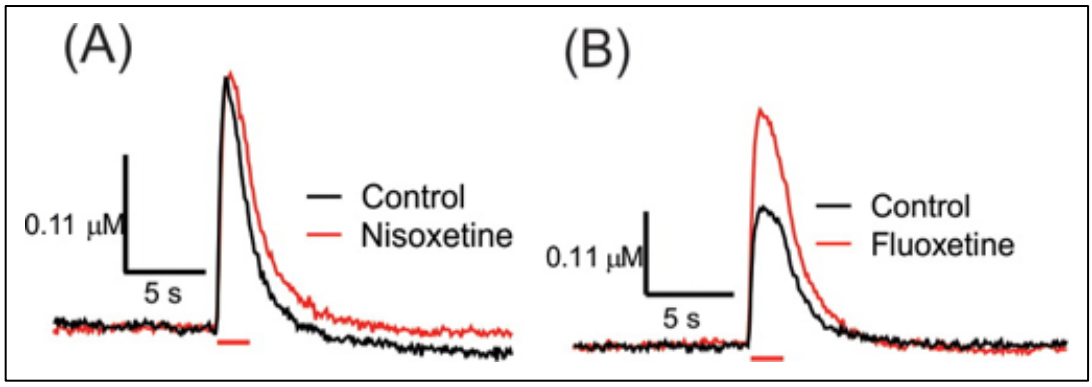

Figure 4.7. Effect of uptake inhibitors. Example concentration vs time plots for 1 s CsChrimson stimulation predrug (black traces) and after 15 min in (A) 20 µM nisoxetine or (B) 100 µM fluoxetine (red traces).

Table 1. Effect of uptake inhibitors on octopamine release and clearance. Effect of dopamine transporter inhibitor (nisoxetine) and serotonin transporter inhibitor (fluoxetine) on evoked concentration and clearance (t_{50}) of octopamine (paired t-test). Values are reported as mean ± SEM

Drug	Value	Before Drug	After Drug	P value
Nisoxetine, n = 5	t_{50} (s)	1.4 ± 0.1	1.7 ± 0.1	$p = 0.1577$
Fluoxetine, n = 7	t_{50}(s)	1.4 ± 0.1	1.4 ± 0.1	$p = 0.4830$
Nisoxetine, n = 5	Concentration (μM)	0.16 ± 0.02	0.18 ± 0.01	$p = 0.4840$
Fluoxetine, n = 7	Concentration (μM)	0.14 ± 0.01	0.20 ± 0.03	$p = 0.0078$**

The t_{50} value of 1.4 s for octopamine is lower than that for serotonin (~ 2.5 s).[38] The rate of diffusion will be similar for both neurotransmitters as they are in similar environment. The small t_{50} value, therefore, suggests that octopamine is either metabolized quickly or transported into cells by some other transporters. N-

acetylation is the major metabolic pathway for neurotransmitter inactivation in *Drosophila*, but metabolism is expected to act on a slower time scale than reuptake.[45] Genome wide searches for transporter DNA have not found any other transporters for octopamine, so future research into mechanisms of clearance in *Drosophila* would be valuable.[39]

Although the t_{50} values were not significantly different in the presence of the transporter inhibitors, evoked octopamine release was significantly higher in the presence of fluoxetine (paired t-test, $^{**}p = 0.0078$, n = 7) (Table 1). This result is similar to previous observations of increased norepinephrine in the presence of fluoxetine in rat brains, and is another indication for the analogy between norepinephrine in vertebrates and octopamine in invertebrates.[44] The increase of norepinephrine in rat brains was 5-HT_{2C} receptor mediated, because fluoxetine is also a 5-HT_{2C} antagonist. However, there is no 5-HT_{2C} receptor homolog reported in *Drosophila.* Other 5-HT receptor homologs have been identified, such as Dm5-$HT_{2\alpha}$, but it has pharmacological properties similar to 5-HT_{2B} receptor in mammals and has not been identified as a 5-HT_{2C} receptor homolog.[46, 47] These results suggest that future experiments might examine the ability of serotonin receptors to mediate increases in octopamine in *Drosophila.*

4.3.7 Comparison of octopamine release with dopamine and serotonin

The concentration of octopamine released with 2 s red light stimulation (0.22 ± 0.03 μM) is slightly lower than stimulated dopamine or serotonin release in similar preparations.[24, 34] For both serotonin and dopamine release, a 2 s

continuous light stimulation produces a peak that after the stimulation ends, but for octopamine, the peak release begins to decrease during the stimulation for a 1 s continuous stimulation. These results suggest a smaller octopamine releasable pool, which was unexpected as octopamine tissue content is higher than both serotonin or dopamine in the larval VNCs.[17] There are several reasons why the stimulated octopamine release could be lower than expected. First, the lower release could mean that *Tdc2-GAL4* is not as strong a driver as that used for dopaminergic (*TH-GAL4*) and serotoninergic (*TPH-GAL4*) neurons. The *Tdc2-GAL4* line used here (Bloomington fly stock 9313) was not made utilizing the site-specificity of attB and attP on DNA and could have resulted in less expression of the driver and thus CsChrimson expression would be lower.[48] Second, octopamine transporters have not been identified in *Drosophila* and the lower release could be because there is no rapid recycling of octopamine through transporters to continually replenish the releasable pool.[39] Finally, the release of octopamine was measured in the neuropil of the VNC, but the GFP images (Fig. 4.1) indicate that there may not be as much arborization and terminal as there are for dopaminergic or serotonergic terminals in that region. Future studies could examine regional differences in octopamine release both in the larval and also adult CNS of *Drosophila*.

4.4. Conclusions

We have detected and quantified stimulated endogenous octopamine release with FSCV for the first time. Optogenetic stimulation or activation of an exogenous ATP channel was used to stimulate vesicular octopamine release.

Release was stable with 2 or 5 minutes interstimulation times, and dependent on the frequency of the applied light. Long stimulations cause decreasing response, so stimulations of 1 s or shorter should be used in future studies. Although the clearance of octopamine was rapid, there was no evidence of octopamine transport through dopamine or serotonin transporters suggesting an alternative method of clearance. The serotonin transporter inhibitor, fluoxetine, did increase the amount of octopamine release, suggesting that future studies are needed to investigate the effect of serotonin on octopamine release. Overall, FSCV can be used to measure octopamine release and clearance rates in larvae, providing insight into the mechanisms of action of this invertebrate neurotransmitter.

4.5. Experimental Section

4.5.1 Chemicals

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), and solutions were prepared in Milli Q water (Millipore, Billerica, MA) unless noted otherwise. Electrode calibrations were performed in phosphate buffer solution (PBS) (131.25 mM NaCl, 3.0 mM KCl, 10.0 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.0 mM Na_2SO_4, and 1.2 mM $CaCl_2$) with pH adjusted to 7.4, which was made once a month and stored at 4°C. To make the larval dissection buffer, 11.1 mM glucose and 5.3 mM trehalose were added to the PBS buffer on the day of the experiment. For $P2X_2$ experiments, ATP solution (0.1 mM) was also prepared daily in the dissection buffer. Ten mM stock solutions of octopamine were prepared in 0.1 M $HClO_4$ once a month and stored at 4°C. Diluted octopamine

solutions for electrode calibration and pressure ejection experiments were prepared daily in PBS buffer.

Disulfiram (Tocris Bioscience, Ellisville, MO) and reserpine (Sigma-Aldrich, St. Louis, MO) stock solutions of 10 mM were prepared in DMSO, and diluted to 400 µM in PBS. Fluoxetine (400 µM) and nisoxetine (80 µM) were prepared in PBS. To add drug to the VNC, 1 mL of the respective solution was added to the Petri dish that contained 3 mL of dissection buffer.

4.5.2 Drosophila and VNC preparation

Drosophila melanogaster stocks were maintained and crossed as described before.[24] Males with *Tdc2-GAL4* on the second chromosome (Bloomington fly stock #9313, gift of Jay Hirsh, University of Virginia) were used to express the following UAS constructs (on the third chromosome): *UAS-CsChrimson* and *UAS-P2X$_2$* (gifts of Vivek Jayaraman, Janelia Farm Research Campus) by crossing *Tdc2-GAL4* males with respective virgin females. Resulting *tdc2-GAL4; UAS-CsChrimson* larvae were fed standard cornmeal food with 400 µM all-trans-retinal and kept in the dark. Larvae were dissected in phosphate buffer and prepared using previously described procedures.[24] Briefly, the central nervous system was dissected out from a third instar larva using fine tweezers, the optic lobes cut off, and the VNC was transferred to the lid of a Petri dish with 3 mL of the dissection buffer in it. For ATP stimulation and octopamine pressure ejection studies, the opposite end of the VNC was cut with fine scissors to facilitate micropipette insertion as described previously.[49]

4.5.3 Electrochemical setup, light stimulation, and data analysis

Carbon-fiber microelectrodes (CFMEs) were fabricated with 7 μm diameter T-650 carbon fibers (Cytec Engineering Materials, West Patterson, NJ) in a 1.2 mm o.d. glass capillary (A-M systems, Carlsburg, WA) pulled to a tip. CFMEs were cut to 50 - 75 μm to form cylindrical electrodes. Data were collected with Dagan Chem-Clamp potentiostat (Dagan, Minneapolis, MN, n = 0.01 headstage), PCI 6711 and 6052 computer interface cards (National Instruments, Austin, TX) and a home-built breakout box. Tar Heel CV software (gift of Mark Wightman, University of North Carolina) was used for data collection and analysis. Electrodes were precalibrated with 1 μM octopamine in a flow cell. Electrodes were also calibrated in the presence of disulfiram and reserpine in the buffer to test any effects on sensitivity. There was no significant effect of either disulfiram or reserpine on electrode sensitivity (paired t-test, $p > 0.1$, n = 4)

The VNC and electrode were viewed under a 40X water immersion lens (Carl Zeiss Microscopy, LLC, Thornwood, NY, USA) and the electrode was inserted in the neuropil from the second to forth abdominal section using a micromanipulator (Narshige N-MMN-1 and N-MMO-202ND). The potential was applied between the CFME and a Ag/AgCl reference electrode in the bath. Optical stimulation was performed with 617 nm LED (~0.2 mW) and driver (ThorLabs, Newton, NJ) with optical fiber placed ~400 μm from the VNC.

A picospritzing pipette was made with the same glass capillary and vertical puller used to fabricate CFMEs. After being pulled, the pipette tips were

trimmed and polished to a 30° angle using a microelectrode beveller (Sutter Instrument Co., Novato, CA, USA). The pipette was filled with 0.1 mM ATP and inserted into the VNC to pressure eject ATP using Picospritzer III instrument (Parker Hannifin, Fairfield, NJ). The pipette was calibrated by measuring the diameter of liquid ejected in oil at a set pressure and ejection time. A similar procedure was used for pressure ejection of 20 μM octopamine.

All statistics were performed with GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Significance was measured with a 95% confidence interval. Graphs are shown as mean ± standard error of the mean.

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Chapter 5: Conclusions and Future Directions

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This thesis describes different methods to stimulate neurotransmitter release in *Drosophila melanogaster* CNS and ways to optimize the FSCV waveform for electrochemical detection of a neurotransmitter. In the second chapter, I described a new method of using acetylcholine and other nAChR agonists to stimulate dopamine release in *Drosophila melanogaster* larval ventral nerve cord (VNC). Acetylcholine stimulates endogenous channels and enables studies of *Drosophila* that don't express any transgenic channels. Mutations in nAChR subunits affected neonicotinoid-stimulated dopamine release without altering the response to nicotine. Therefore our method allows studies of how mutations affect the neurochemical properties of nAChRs, which are important targets for insecticides. Using the new stimulation method, evoked dopamine was also measured in adult *Drosophila* brains, as described in Chapter 3. I studied stimulated dopamine release in control and Parkinson's disease model adult *Drosophila* at different ages. This was the first time we detected evoked dopamine in adults. Our lab has tried to study optogenetically-stimulated dopamine release in transgenic adults with little success. My work, therefore, makes it possible to study dopamine neurotransmission in adult *Drosophila melanogaster.* The fourth chapter describes optimization of a FSCV waveform for octopamine detection *in situ*, and characterization of stimulated octopamine in *Drosophila* larval VNC. Octopamine is a major insect neurotransmitter that is analogous to norepinephrine in mammals. It oxidizes at a higher potential than dopamine and the oxidation peak appears close to the switching potential with

the traditional dopamine waveform. Also, octopamine is a phenol and its oxidation products form polymers that foul the electrode surface. Detection of octopamine in *Drosophila* has been hampered due to these limitations. With the FSCV waveform I have optimized, we can now detect and quantitate evoked octopamine. This enables the study of octopamine neurotransmission and how it changes in disease models or in mutant lines.

In this final chapter, I summarize the main conclusions and suggest future directions for the field. The work described in this thesis allows the study of stimulated dopamine release in most *Drosophila* lines. Previous research required the expression of transgenic channels to study dopamine release, but that is no longer a limitation. The method has also allowed us to move into adults and study changes of dopamine neurochemistry with aging and in flies that model age-related human disease. Finally, the method developed for the detection of octopamine *in situ* will enable future studies of the neurochemistry of this important invertebrate neurotransmitter.

5.1. nAChR mediated dopamine release in *Drosophila melanogaster*

In the second chapter, I have described how nAChR agonists stimulate dopamine release in *Drosophila* larvae. In previous studies, neurotransmitter release was mediated by exogenous channels expressed in either dopaminergic or serotonergic cells.[1-3] The channels were activated by light (ChR2 or CsChrimson) or ATP ($P2X_2$). While expression of transgenes is relatively simple in *Drosophila,* it can be a disadvantage when lines that don't express the

channels need to be tested or when many genes must be expressed simultaneously. For example, the *Parkin-RNAi* flies have a mutation for *Parkin* on their third chromosome and to insert the exogenous channel, it must be expressed using a different chromosome and balancers. Therefore, we developed a method to evoke endogenous neurotransmitter release in flies that don't express special channels.

Since acetylcholine stimulation evokes endogenous dopamine by activating endogenous nAChR channels, the method can be used to study release in wild type flies or disease mutants that do no express exogenous ion channels. We have previously not been able to detect optogenetically-stimulated dopamine in adult *Drosophila* flies (due to low expression of the channel), but can now study it with acetylcholine stimulation as described in Chapter 3. This method is very useful for initial screening of mutations that could affect dopamine; we can take any fly and easily measure the acetylcholine-stimulated release at any age without having to make any mutations. For example, we can study effects of various disease mutations, such as mutations of different genes associated with Parkinson's disease and how they affect dopamine release, clearance and dynamics in the brain. Thus, we expect many preliminary studies to see the effects of genes on neurochemistry that will be performed with this method in the future.

We used acetylcholine, nicotine, or neonicotinoids to stimulate dopamine release in wild type *Drosophila* larval VNC. We discovered that nicotine and

neonicotinoids evoke release that last longer than release evoked by acetylcholine. This provides evidence for the mechanism of how nicotine and neonicotinoids act as insecticides by overstimulating nAChRs. Our method allows studies of the effects of neonicotinoids on the neurochemistry of insects and can be extended to mammals to understand the extent to which neonicotinoids affect mammalian nAChRs. This can also aid in the discovery of neonicotinoids that affect insects but only minimally affect mammals. The possibility to study neurochemical effects of nAChR and its subunit mutations on neonicotinoid evoked responses also opens doors to use the *Drosophila* model organism for rapid screening of nAChR subunit mutations that contribute to insecticide resistance.

nAChRs are important insecticide targets, and insect nAChRs are difficult to express in host cells.[4] In flies that have mutations in the nAChR subunits and are resistant to the neonicotinoids, there was no neonicotinoid-stimulated release, which suggests that mechanisms for stimulated dopamine release and resistance development are linked. This validates the use of our method to study the neurochemical effects of nAChR mutations, and future use of *Drosophila* to understand insecticide resistance better. *Drosophila melanogaster* is a widely used model organism to study the nervous system and there are several toolkits available for its genetic manipulation. Our method enables investigations of how small changes in the nAChRs affect their sensitivity to various agonists including neonicotinoids. Future studies can investigate how dopamine release and

clearance are affected by changes in the nAChR subunits, which will lead to better understanding of target site specificity of agonists. Neurochemical measurements in *Drosophila* can therefore be used to understand neonicotinoid resistance development to develop better resistance management methods.

Another area of investigation that my research enables is the study of effects of nicotine on nAChRs. Nicotine causes dopamine release in the brains in mammals, and I show here that there is dopamine response in *Drosophila* as well. How different nAChR subunits play a role in addiction and withdrawal symptoms in humans is not well understood. Since dopamine in involved in the reward pathway, the method I described here can be used to understand the effects of different subunit mutations in regulating dopamine dynamics. Future research can investigate how changes in the different α and β subunit affect dopamine response in *Drosophila,* and provide an understanding of how addiction and withdrawal symptoms can be managed better.

5.2. Evoked dopamine release in adult *Drosophila* and in Parkinson's disease model

As discussed earlier, we have extensively studied stimulated neurotransmitter release in *Drosophila* larval VNC in our lab.[1-3] For the first time, we studied evoked dopamine release in adult *Drosophila* brains with acetylcholine stimulation. The central complex region of the adult brain is rich in dopaminergic projections and we measured stimulated dopamine release in the central complex.[5-8] Evoked release increased with increasing amounts of

acetylcholine stimulation, and plateaued at 10 pmol acetylcholine. The tissue content of dopamine in the CNS was also measured with capillary electrophoresis (CE). The effect of aging on dopamine was studied at three different ages in adult *Drosophila* control and *Drosophila* with *Parkin-RNAi* or *RNF11-RNAi* mutations.

The effect of aging on dopamine was first studied in control *Drosophila* to establish baseline conditions for studies in Parkinson's disease mutant flies. There was no significant effect of age on evoked dopamine or on dopamine tissue content. This result was consistent with a previous study that reported no change in the expression of TH in the brains of *Drosophila* as they aged.[9] There was a significant effect of age when stimulations were repeated at 2-minute intervals as old flies had less decrease of evoked dopamine than mid-age flies. The result shows that unlike human brains where there is a decline in dopaminergic cell bodies and TH expression with age, *Drosophila melanogaster* do not have a significant change of dopamine levels in the CNS with age. The result is surprising as whole body levels of dopamine decline with age in *Drosophila*.[10] Future studies should investigate if there is a decline of dopamine tissue content or stimulated release of dopamine in specific regions of the CNS. I established the baseline levels of dopamine tissue content and stimulated release with aging in control flies, and this method enables future studies of effects of aging in *Drosophila* models of age-related diseases such as Parkinson's disease and Alzheimer's disease.

Next, effects of aging on dopamine in *Parkin-RNAi* or *RNF11-RNAi* *Drosophila* were investigated. Mutant *Parkin* is associated with human familial cases of Parkinson's disease and when expressed in *Drosophila,* it causes dopaminergic cell loss in *Drosophila* neurons.[11] Also *Parkin* null mutant *Drosophila* have shrinkage of a subset of dopaminergic cells.[12] *RNF11* is another gene involved in Parkinson's disease, and is thought to be neuroprotective.[13, 14] *Parkin-RNAi* flies tested here did not have a significant change in dopamine tissue content with age, but *RNF11-RNAi* showed a significantly higher dopamine tissue content at old age. Both lines showed no significant difference in evoked dopamine release when stimulations were repeated at 2-minute intervals. This was different than control flies where old flies showed significantly less decrease during repeated stimulations compared to mid-age flies. The control flies therefore replenish more of the releasable pool of dopamine as they age, and the effect is lost in the Parkinson's disease model *Drosophila.*

Our study provides evidence that knocking down *Parkin* with RNAi causes no change in the concentration of stimulated dopamine release in the central complex or dopamine tissue content in the CNS of *Drosophila,* but does change dopamine dynamics at old age. Future studies should investigate if completely knocking out the *Parkin* or expressing human *Parkin* mutant gene has effects on dopamine levels. *Parkin* knockout flies have given mixed results on dopaminergic cell loss, and future studies of mutants that show a definite loss of dopaminergic cells will be tested to see if there is a significant defect in dopamine release and

tissue content. [11, 15, 16] *Parkin* null mutants with a complete knockout of Parkin have shown an effect in dopaminergic cells, however the effect of stimulated release is not studied.[12] As my study sets a foundation for studying dopamine in adult *Drosophila,* it allows future investigations of changes in dopamine dynamics in *Parkin* null mutants that have previously shown to have smaller dopaminergic cells.[12] Future research can therefore examine the effects of different Parkinson's disease mutations on the levels of dopamine and stimulated dopamine concentration in different brain regions. For example, two separate studies of *Parkin* mutants have reported dopaminergic cell loss in the PPL2ab or PAM regions.[17, 18] The PPL2ab and PAM regions and regions where they send their projections, the calyx and the horizontal lobes, respectively, can be first investigated for any changes of dopamine signaling due to *Parkin* mutations. The study will advance the knowledge of how different brain regions are affected in Parkinson's disease model *Drosophila* and enable future studies of effects of drug treatments on dopamine neurochemistry.

There is no clear evidence of how much reduced TH expression in flies correlates with dopaminergic cell death or neuronal dysfunction.[19] Direct measurements of levels of dopamine and tissue content with our method will allow better understanding of neurochemical changes in dopaminergic cells. Similarly, dopamine levels in *Drosophila* models of Alzheimer's and Huntington's disease can also be investigated as dopamine is suggested to play a role in the pathology of these diseases.[20, 21] Thus the method described here enables future

studies of dopamine neurochemistry in *Drosophila* Parkinson's disease models as well as other disease models.

5.3. Optimization of a FSCV waveform for in situ detection of octopamine

The fourth chapter of this thesis describes optimization of a FSCV waveform for the detection of octopamine *in situ.* Octopamine is a major invertebrate neurotransmitter and plays important roles in learning, memory formation,[22] locomotion[23] and ovulation.[24] Our lab had studied evoked dopamine and serotonin release in *Drosophila melanogaster*, but was not able to study octopamine.[1, 2] A FSCV waveform was optimized for *in vitro* detection of octopamine,[25] but the waveform was not suitable *in situ*. Octopamine oxidizes at a higher potential than dopamine or serotonin and the oxidation peak is shifted to the right close to the switching potential. The fast scan rate (600 V/s) of the waveform optimized for *in vitro* octopamine detection and slowed electron transfer process *in situ* caused the peak shift to be even greater.[26] Switching potential is where the signal is most unstable and non-faradaic signals can interfere with peaks close to the potential. By scanning up to 1.4 V and at a slower potential of 100 V/s, I was able to detect and characterize stimulated octopamine release in *Drosophila* larval VNC.[27]

Octopamine is a phenol and its oxidation product can further oxidize and create polymers at the electrode surface. This fouls the electrode and cause unstable responses. The new slower scan waveform lowered the effect of fouling by octopamine oxidation products as stable current responses were observed

with repeated stimulations both *in vitro* and *in situ.* The method can be extended to other species that can cause electrode fouling, such as serotonin and tyramine.[28, 29] FSCV waveform optimization described here can also be used in the future to increase the sensitivity for other analytes or to increase selectivity towards a particular analyte.

Octopamine detection *in vivo* opens doors to study the neurochemical signaling of this molecule by FSCV. For example, we used the new waveform to show that *RNF11-RNAi* mutation in *Drosophila* larvae causes an increase in stimulated dopamine release, but not octopamine release, which shows that the mutation affects dopaminergic cells specifically.[30] We can also study effects of other mutations such as ones in *Drosophila* vesicular monoamine transporter (dVMAT), and how they affect stimulated octopamine release with the method I have developed.[31] Mutations in dVMAT affect larval locomotion or female fertility, both behaviors that are also modulated by octopamine. Therefore, future research can investigate how octopamine release and clearance kinetics are affected in dVMAT mutants to understand the link between behavior and octopamine neurochemistry. Optogenetically-stimulated octopamine release at the *Drosophila* neuromuscular junctions (NMJ) has been studied with amperometry as octopamine modulates the NMJ in arthopods.[32] Amperometry is not a selective method as FSCV, and our method will allow selective measurements of evoked release in the NMJ in the future.

Being able to detect endogenous octopamine *in situ* will allow better understanding of how octopamine neurotransmission mediates learning, memory, locomotion and ovulation. Future studies can also investigate if acetylcholine-stimulated octopamine release can be measured in *Drosophila.* Octopamine release mediated by nicotine has been measured in *Drosophila* before and since both nicotine and acetylcholine act on nAChRs, we expect to measure acetylcholine-stimulated octopamine release as well.[33] The previous study used chronoamperometry to measure nicotine-stimulated octopamine release, and with FSCV we can make more selective measurements of the release. Our method will enable studies of stimulated octopamine release in any fly as release will be mediated by endogenous channels.

5.4. Final Remarks

This dissertation describes new methods to study stimulated dopamine release in *Drosophila melanogaster* larval and adult CNS. nAChR mediated dopamine release was measured in *Drosophila* larva and significant effects of nAChR mutations in neonicotinoid evoked response was measured. The study enables investigations of how nAChR mutations can mediate neurochemical responses in insects. Since nAChRs are natural ion channels, the new stimulation method can evoke dopamine release in wild type flies and other mutant flies that do not express special ion channels. For example, the method was used to study evoked dopamine release in adult *Drosophila.* Effects of aging on dopamine release in control flies and flies with Parkinson's disease mutations

were also studied using acetylcholine stimulation. There was a significant effect of age when stimulations were repeated at short time intervals, and the effect was lost in Parkinson's disease mutant flies. This suggests that Parkinson's disease mutations affect how fast the releasable pool of dopamine restores in old adults. The study also establishes a baseline of dopamine signaling for aging studies in *Drosophila.* Future studies should investigate how dopamine dynamics change with age in *Parkin* null mutants and *Drosophila* expressing mutant human *Parkin* genes to see if the effects in dopamine neurochemistry are greater than with *Parkin* knockdown mutations. This thesis also describes optimization of the FSCV waveform for octopamine detection in *Drosophila* larval VNC. The waveform was optimized to decrease the effects of electrode fouling by oxidation products of octopamine for stable measurements of octopamine *in situ.* Red light stimulated octopamine in larval VNC was characterized. The research enables future studies of octopamine in *Drosophila*, such as investigations of how mutations in *Drosophila* affect octopamine neurotransmission. Similar methods of FSCV waveform optimization can be investigated for electroactive compounds that give unstable responses. FSCV allows rapid, sensitive and selective measurements of neurotransmitters. I have described methods here that can measure dopamine and octopamine in *Drosophila* larvae and adult CNS. The methods will enable future studies of how these neurotransmitters affect different functions in *Drosophila,* and how different mutations and disease models affect the neurotransmitters.

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