NEUROLOGICAL ANALYSES OF
*DROSOPHILA MELANOGASTER* WITH
CAPILLARY ELECTROPHORESIS

A Thesis in
Chemistry

by

Nicholas John Kuklinski

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The dissertation of Nicholas John Kuklinski was reviewed and approved* by the following:

Andrew G. Ewing  
Professor of Chemistry  
J. Lloyd Huck Chair in Natural Sciences  
Dissertation Advisor  
Chair of Committee

Anne M. Andrews  
Associate Professor of Molecular Toxicology

Peter J. Butler  
Associate Professor of Bioengineering

Michael T. Green  
Associate Professor of Chemistry

Barbara J. Garrison  
Shapiro Professor of Chemistry  
Head of the Department of Chemistry

*Signatures are on file in the Graduate School
ABSTRACT

This thesis highlights the development and optimization of capillary electrophoresis (CE) based techniques for neurological analyses of the fruit fly *Drosophila melanogaster*. Fast separation times (seconds to minutes), minimal sample requirements (nanoliters to femtoliters), and excellent mass detection limits (femtomole to zeptomole) make CE ideally suited for sampling of neuromodulators with a high degree of spatial resolution. Significant progress has been made to resolve amines and improve upon sampling techniques used to investigate these complex biological systems.

Micellar electrokinetic chromatography (MEKC) coupled to amperometric electrochemical detection (EC) was used to resolve and then quantify biogenic amines and metabolites within *Drosophila melanogaster*. In Chapter 2, a new separation scheme was devised to allow resolution of 24 compounds of interest. This was accomplished by precisely controlling the amount of base added to the background borate/ sodium dodecyl sulfate (SDS) buffer, which optimizes the resolution of the separation, and then calculating the pH. Here I focused on measurements of six of the analytes that are thought to be involved in the response to alcohol: dopamine, salsolinol, norsalsolinol, N-acetyloctopamine, octopamine, and N-acetyldopamine. These were identified and quantified within homogenates of the fly head. We believe the identification of salsolinol and norsalsolinol in the fly brain is novel.

I then used the separation scheme that I developed in Chapter 2 to quantify biogenic amines within individually, microdissected *Drosophila melanogaster* brains and brain regions in Chapter 3. The effects of pigment from the relatively large fly eyes on...
the separation were examined to find that the red pigment from the compound eye masks much of the signal from biogenic amines. The brains of white mutant flies, which have characteristically low pigment in the eyes, have a significantly simplified separation profile in comparison to the red-eyed, wild-type, Canton S fly. Yet, the white mutant flies were found to have significantly less amounts of dopamine, L-3,4-dihydroxyphenylalanine (L-DOPA), salsolinol, and N-acetyltysamine in their dissected brains when compared to dissected brains of Canton S flies. In addition, significant variation was observed in the dissected brains between individual flies that might be related to changes in neurotransmitter turnover. The transgenic GFP fly line (TH-GFP), for which the overall profile of biogenic amines is not found to be significantly different from Canton S, was then used to visualize the location of and dissect dopamine neurons. Biogenic amines were then quantified in three brain regions observed to have dopamine levels, the central brain, optic lobes, and posterior superiormedial protocerebrum (PPM1) region.

Microdissection techniques were expanded in Chapter 4 to analyze population homogenates fly brains through the process of freeze-drying, a dehydration process where a material is quickly frozen, has its surrounding pressure reduced, and is then heated to allow sublimation of the water from the sample. Extraction and drying times were quantitatively explored and optimized. Drying for too long produced low signals possibly due to sample loss, while long freezer storage times led to over-extracted samples resulting in unidentified and undesirable electroactive compounds from the eyes of the fly. Freeze-drying fly heads makes the outer walls of their cuticles hard and brittle while also freeing the brain from the inner walls of the cuticle. This significantly
decreased the time necessary to dissect a fly brain and afforded larger numbers of brains to be homogenized to make a sample. The number of brains in a homogenate was increased, effectively concentrating the sample, and helped to increase the amount of signal and resolution seen. Biogenic amines were then quantified in samples containing fifteen freeze-dried brains. N-acetylserotonin, N-acetyltarotamine, N-acetyldopamine, L-DOPA, and tyramine were discovered to all correspond well with previous dissected studies.

CE-EC is a powerful technique for the analysis of neuromodulators offering low limits of detection and high selectivity against background signals in biological samples. Yet, this selectivity of electroactive metabolites and transmitters prevents the analysis of many other neurological analytes of interest. Chapter 5 presents the development of separations using CE electrospray ionization (ESI) time-of-flight (TOF) mass spectrometry (MS) for the analysis of neurotransmitters, metabolites, and drugs within the brains of Drosophila. Optimizing a volatile buffer at a specific pH ensured a unique m/z ratio is available for each analyte when they are detected.
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PREFACE

Portions of this thesis are the result of collaborative work that has been adapted for publication in peer-reviewed journals:


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Chapter 1

Chemical Measurements in *Drosophila*

**Introduction**

*Drosophila melanogaster*, the fruit fly, is an attractive model system with experimental and genetic qualities that have made it a prominent tool in developmental biology and genetics research. Originally pioneered by Thomas H. Morgan at the beginning of the last century, research using *Drosophila* has led to important insights into the mechanisms of human developmental and physiological processes as evolutionary conservation between the two species has made the fly a successful model organism. Although the adult fly has a more simple nervous system when compared to vertebrates, it is capable of higher-order brain functions including both aversive and appetitive learning and recalling learned information from prior experiences.\(^3\)\(^,\)\(^4\) In addition, *Drosophila* larvae can be used as a model for investigating basic neurotransmission and chemosensory pathways\(^5\) as the conservation between the *Drosophila* and mammalian proteomes is high with approximately half the protein sequences in the fly having similar counterparts in the human sequence. The fly matures relatively quickly, developing from an embryo, to larva (divided into 1\(^{st}\), 2\(^{nd}\), and 3\(^{rd}\) instar larva stages), to pupa, to a sexually mature adult in a span of ~12 days, making the fly an attractive model when compared to longer to mature mammalian models. However, an adult fly brain is approximately 5-nL in volume and comprised of several distinct structures which control
specific tasks. These very small dimensions are a challenge for researchers attempting chemical quantification in the fly and necessitate the use of techniques capable of handling mass-limited samples.

Studies of neurotransmitters are underway in *Drosophila* to elucidate the roles of neurochemicals in human behavior. Biogenic amines, namely dopamine, serotonin, and tyramine, are known to be involved in physiological processes found in both mammalian and *Drosophila* systems. For example, dopamine has been implicated in human and fly behaviors such as reward and motivation, sleep cycles, alcohol tolerance, and sensitivity to addictive drugs. Tools that fluorescently label specific neuron clusters in the brain are available (Figure 1-1B) which has made recent real-time, sensitive in vivo measurements of dopamine uptake by dopaminergic neurons in *Drosophila* possible. In addition, the neurotransmitter octopamine is thought to control many behaviors in the fly that norepinephrine regulates in mammals. These studies suggest that many of the
neurotransmitter systems that regulate behavior are comparable between mammals and *Drosophila*.

The *Drosophila* proteome has been a natural choice to study, as it was one of the first species with a fully sequenced genome.\textsuperscript{16} The process of producing mutants to display a desired behavior via genetic manipulation is a relatively straightforward task with the fruit fly. The *Drosophila* genome contains little genetic redundancy, or multiple genes performing the same biochemical function, which facilitates identification of individual genes and molecules that influence a particular behavior.\textsuperscript{1} Many complex behavioral patterns found in mammalian systems with regards to learning and memory, courtship, alcohol tolerance, and circadian rhythms have been studied in the fruit fly through the use of genetic mutants.\textsuperscript{17-20}

*Drosophila* mutants have been successfully used to model several human neurodegenerative diseases, including Alzheimer’s disease, Parkinson’s disease, and Huntington’s disease. These diseases are characterized by the late onset of progressive neurodegeneration and/or formation of abnormal neuronal inclusions or protein aggregates.\textsuperscript{21,22} While these genetic mutants have helped in linking particular genes to a specific disease, little is known about the mechanisms leading up to these pathologies. The ability to quantify all neuropeptides, amino acids, and neurotransmitters in *Drosophila* is a goal researchers are moving towards and will lead to a greater understanding of these debilitating diseases. Obtaining spatiotemporal information along with chemical quantification will help provide a more analytical view of *Drosophila* and will likely lead to an improved understanding of the physiological mechanisms that underlie human behaviors, addictions, and neurodegenerative diseases.
Analytical techniques to measure physiology of intact flies

Recently, analytical tools have been developed to record chemical measurements in adult *Drosophila* and *Drosophila* larvae in real-time. The ability to acquire direct physiological information will bridge the gap between observed fly behavior and the chemical signaling pathways that underlie those behaviors. Work has also been done to develop technologies for manipulating individual *Drosophila* embryos to study development, and these tools will enable us to answer questions about the functions of individual organisms.

Individual adult fly measurements

Electrochemical detection has been used for *in vivo* measurements of electroactive biogenic amines in model systems such as rats, mice, and primates, but until very recently these measurements were not feasible in an organism as small as *Drosophila*. The Ewing laboratory has developed a method to monitor dopamine in the fly CNS using background-subtracted fast-scan cyclic voltammetry (FSCV) with carbon-fiber microelectrodes. This method has been successfully used to investigate uptake of exogenously applied dopamine by the *Drosophila* dopamine transporter (DAT). Figure 1-2A,B compares the concentration of dopamine in a wild-type fly vs. a *fumin* (*fmn*) mutant fly measured before and after treatment with cocaine. This drug of abuse is known to block the uptake of dopamine by the DAT impeding the uptake of DA. The *fmn* lacks a functional DAT, and thus uptake should not be affected. The data shows that the peak dopamine concentration, [DA]$_\text{max}$, increased 3-fold after cocaine treatment for
the wild-type fly, uptake remained unchanged for the fmn mutant. When the average [DA]_{max} of multiple flies was considered (Figure 1-2C\textsuperscript{13}), the [DA]_{max} of untreated wild-type flies was significantly lower than for fmn mutant flies. Interestingly, the [DA]_{max} for
cocaine treated wild-type flies was not significantly different from the untreated fmnn mutant flies which supports existing evidence that cocaine effectively blocks the Drosophila dopamine transporter. These measurements validate the use of this in vivo method in flies as a model system to study drug addiction mechanisms.

In addition to electrochemical techniques, calcium imaging has been employed for making intact, whole fly measurements using genetically encoded fluorescent proteins. Fluorescent proteins that measure calcium changes (an accepted indicator of electrical activity) can be genetically expressed in specific neurons to target a tissue of interest in the Drosophila brain using the GAL4-UAS system. This methodology has been explored using several calcium-sensitive fluorescent proteins including cameleon 2.1, camgaroo 2, and G-CaMP. Fiala and coworkers have labeled the mushroom body calyx and antennal lobe in the Drosophila CNS with cameleon 2.1 and measured odor-evoked calcium signals in vivo from both regions. Moreover, this technique can be altered to target any brain region of interest for which a GAL4 “driver line” exists. Based upon previously published work on dissected mushroom bodies by Davis and coworkers, the GAL4-UAS system was used to label the mushroom bodies with camgaroo 2, and the intensity changes of the fluorescent Ca²⁺ reporter in response to acetylcholine application were recorded in an intact fly. Axel and colleagues employed two-photon calcium microscopy to image the antennae lobes of flies that expressed G-CaMP in their projection neurons. Using this technique, they were able to link odor-induced calcium changes to specific regions of the antennal lobe. Each odor elicited a distinct pattern, which appears to be conserved between different organisms of the same fly line. These calcium-imaging techniques could potentially be used for
quantitative investigation of olfactory learning and memory in the *Drosophila* mushroom bodies and antennae lobes.

**Individual larva measurements**

There has been recent progress in the development of techniques for measuring neurotransmitters from individual *Drosophila* larvae using a combination of electrochemical detection and optical stimulation methods (Figure 1-3A). Channelrhodopsin-2 is a light-activated cation-selective ion channel that when placed under the control of a GAL4-UAS system and crossed with flies of a “driver line” specific to serotonin (Tph-GAL4), will produce larvae that release serotonin upon exposure to blue light. Recently, Venton and colleagues utilized these transgenic larvae to measure serotonin release from neurons located in an isolated larva ventral nerve cord (VNC) using FSCV with a microelectrode. The extracellular serotonin concentration in the VNC was found to consistently vary between 280-640 nM with the duration of blue light exposure (Figure 1-3B, C). Inhibition of the serotonin transporter with cocaine and fluoxetine confirmed that the removal of serotonin from the extracellular space was due to transport, and demonstrated the potential use of this model system for studying basic serotonin signaling mechanisms.
The *Drosophila* larva \(^{31,32}\) model system has potential use in other areas as well. A novel sampling technique has been developed to obtain nanoliter volumes of hemolymph from individual *Drosophila* larvae for chemical analysis. Hemolymph contains amino acids such as glutamate and glutamine that are thought to play a role in neurodegeneration. This technique extracts 50-300 nL of hemolymph from a single *Drosophila* larva then, following derivatization with fluorescamine, its amino acid

![Diagram of neuromuscular anatomy of a third-instar larva.](image)

**Figure 1-3** *In vivo* measurements in *Drosophila* larvae following optical stimulation. (A) Diagram of neuromuscular anatomy of a third-instar larva. (B) Representative traces of evoked peak serotonin concentration varying with blue light stimulus duration (2, 5, 10, and 30 s). (C) Pooled data (mean ± SEM, \(n=6\)) shows an increase in peak height with increasing duration of blue light exposure. Peak height appears to plateau after 10 s; peak height at 30 s is not significantly different from that at 10 s (Student’s *t*-test, 2 tailed, *p* = 0.78). (Reprinted with permission from the Society for Neuroscience and Elsevier).
content is quantified using CE with laser-induced fluorescence detection. In a demonstration of this technique, Shippy and coworkers compared genderblind (gb) larvae, mutants developed previously by collaborators that contain approximately half the normal extracellular glutamate concentration, to wild-type larvae. Overall the gb mutants were found to have 38% lower glutamate levels than the wild-type larvae with 13 amino acids in total being successfully separated and quantified from each larva’s hemolymph \( (n = 10-17) \). These initial findings support the continued development of this technique in quantifying amino acid levels from individual Drosophila hemolymph to better understand their role in human disease.

**Controlling individual fly embryo development using microfluidics**

There is an increasing interest in using Drosophila embryos to study mechanisms of development and gene function. One powerful method of silencing a gene of interest is called RNA interference (RNAi). Cells are exposed to specifically designed double-stranded RNA (dsRNA) that, once inside the cell, is cleaved into smaller dsRNA pieces (siRNAs) by endogenous enzymes. The siRNA then binds to a RNA-induced silencing complex (RISC) where it becomes unwound. The unwound siRNA guides the RISC to the corresponding messenger RNA (mRNA) whereby the RISC destroys the mRNA, thus eliminating the coding of that particular gene and the gene’s subsequent function. While using cells for high-throughput screens is useful, embryos are more ideal model systems for studying development and gene function because they possess physiological content with greater biological complexity; however, until recently, performing RNAi on
Figure 1-4 Microfluidic devices in the analysis of *Drosophila* embryos. The rate of development in each half of the embryo exposed to a T-step is affected by temperature, as demonstrated by the difference in nuclear density (number of nuclei in enlarged areas shown underneath in yellow numbering). (A, B) Embryos exposed to a T-step of 20 °C/27 °C for 140 min. (A) Anterior half 20 °C, posterior half 27 °C. (B) Anterior half 27 °C, posterior half 20 °C. (C, D) Identical set-up to A and B with embryos exposed to a greater T-step of 17 °C/27 °C for 150 min. In all images, higher nuclear density was observed in the warmer half of the embryo. (Reprinted with permission from Nature Publishing Group).
embryos was a tedious process that required a skilled technician to individually inject each embryo by hand. Solgaard and colleagues have developed a microfluidic device coupled with a computer-controlled injection system to inject *Drosophila* embryos with dsRNA for high-throughput RNAi screens.\textsuperscript{16} This microelectromechanical systems-based device has been automated to detect embryos on a glass slide, followed by rapid injection of 60 pL RNAi aliquots into each embryo with 98% reliability. Although preliminary prototypes require initial manual injector alignment to the device, it has great potential for future development into a fully automated process and has already been adapted for various embryo applications where controlled microinjections of small molecules, such as drugs or proteins, are necessary.\textsuperscript{37, 38}

Microfluidic technologies have also been utilized to develop devices capable of spatial and temporal control of developing *Drosophila* embryos. Ismagilov and coworkers\textsuperscript{39, 40} have used a ‘Y’ junction device to investigate a compensatory regulation mechanism displayed by developing embryos towards external perturbations in temperature (Figure 1-4 top\textsuperscript{39}). When the anterior and posterior sides of an embryo were exposed to an extreme temperature gradient using two laminar streams held at different temperatures, the warmer half of the embryo had a higher number of nuclei, and therefore was developing more rapidly, than the cooler half (Figure 1-4A,B\textsuperscript{39}). When the temperature difference between the anterior and posterior sides was increased from 7°C to 10°C, the difference in the rate of development between the two sides increased as well (Figure 1-4C,D\textsuperscript{39}). Also, the Even-skipped gene (a gene that codes for segmentation during early embryonic development) was expressed sooner in the warmer region of the embryo causing the usual 7-stripe segmentation pattern to develop in the wrong order.
Interestingly, despite the different developmental rates forced upon the two regions of the embryo, when allowed to come to room temperature, the embryos displayed the completed stripe pattern correctly and developed into normal larvae suggesting *Drosophila* have a compensation mechanism to counteract extreme environmental conditions during embryo development. This device has since been adapted to allow for easier attachment of the embryos.\(^{41}\) Continued modifications that enhance the ability to apply external gradients to an immobilized embryo will pave the way for future studies on the mechanisms of biochemical networks during development.

**Detection methods for analysis of *Drosophila* homogenates**

Many methods have been used to separate and quantify mass-limited samples including capillary CE, high-performance liquid chromatography (HPLC), and mass spectrometry (MS). Indeed, these methods are sensitive and selective making them capable of measuring and identifying multiple compounds in a complex biological sample. This ability allows determination of how different stimuli affect neurochemicals within the brain and is crucial to understanding changes throughout disease states.

Historically these analytical techniques have used homogenate methods to contend with the fly’s hard cuticle. Approximately 25-75 whole fly heads can be pulverized using small tissue grinders, or brains (~10) can be hand dissected using tungsten-carbide forceps, collected, and homogenized. While whole fly head samples allow for more flies in the homogenate helping to reduce fly-to-fly variability, it leaves a significant matrix effect that can interfere with quantification. A sample of dissected
brains can help to reduce these interfering signals; however, sample preparation is more
time consuming, requires knowledge of dissection techniques, and limits the number of
flies in a sample.

**High-performance liquid chromatography**

Just as in CE, HPLC has been used to quantify the amount of biogenic amines,
their metabolites, and their precursors in the *Drosophila* CNS. These studies have aimed
to determine the function of molecules and their localization within the fly head. HPLC
is an improved form of column chromatography where solvent is pushed though the
column under high pressures (up to 40 MPa). The high pressure allows for faster
separation times and smaller column particles, yielding improved resolution. Typically, a
C-18 column with an acidic mobile phase and electrochemical detector has been used to
separate and detect these compounds.42-46

Early reports using HPLC demonstrated the separation and quantification of
dopamine, L-DOPA, and α-methyldopa in one to four week old brains and retinas of
wild-type and mutant *ebony* flies, which have a darker pigment and impaired vision.46
Although the levels of all three analytes were variable over time, the authors did report
that these analytes were more abundant in the retina than in the brain and more abundant
in the mutant *ebony* fly heads than the wild-type fly heads.

Hardie and Hirsh expanded the number of neurotransmitters analyzed, quantifying
dopamine, octopamine, tyramine, and serotonin in the brains and whole heads of
*Drosophila* white-eyed (*white*) mutants.42 They noted that nearly 75% of the total
dopamine within the *white* mutant head is located outside of the brain. In contrast, the percentages of octopamine, tyramine, and serotonin present outside of the brain range from only 1 to 37% when compared to the amount in the brain.

HPLC’s quantitative nature also has been utilized to examine the role of tyramine in cocaine sensitization studies of the *inactive* and the *TβH*<sup>M18</sup> *Drosophila* mutants<sup>9</sup> The *inactive* mutant, named for the flies’ low activity levels, was found to have approximately 60% less tyramine than wild-type flies, despite similar measurable levels of dopamine. While these mutants displayed expected behavioral responses to cocaine upon their first exposure, with repeated cocaine exposure minimal behavioral sensitization to cocaine was observed. The *TβH*<sup>M18</sup> line has a null mutation in the gene that codes for tyramine β-hydroxylase, the enzyme used to convert tyramine into octopamine. These flies were found to have almost an order of magnitude greater amount of tyramine and near-normal cocaine sensitization when compared to the wild-type fly, ruling out octopamine as the biogenic amine contributing to this cocaine sensitization. These two comparisons show that tyramine plays a critical role in cocaine sensitization and later helped to confirm the identity of two tyrosine decarboxylase genes.<sup>45</sup>

The Meinertzhagen laboratory has further investigated the location and quantification of biogenic amines within the brain of genetic fly mutants with different eye pigments. They developed a method in the fly to quantify histamine,<sup>43</sup> a transmitter known to be located in the eyes of the fly, and compared it, along with the amount of dopamine and serotonin, in *white, brown,* and *scarlet* mutants, flies with three different eye-pigment mutations.<sup>44</sup> Since scarlet and brown are the two pigments that control fly eye color, knocking out one pigment results in a fly with the other eye color, and a
knockout of both pigments results in no eye pigment, white mutants. They measured a significant decrease (in some cases over 50%) in the neurotransmitters of all three Drosophila mutants when compared to wild-type flies. Similar trends were observed in comparisons of wild-type vs. white mutants of houseflies, blowflies, and two species of the flesh fly, signifying that many effects attributed to a mutant gene isolated in a white fly might be from the loss of pigment itself and not the mutated gene. They also noted that in separations of wild-type fly head homogenates, 71% of the total dopamine in the head was found in the brain, in contrast to Hardie and Hirsh’s results for white mutant flies.

Mass spectrometry to study proteins and peptides

Mass-spectrometric studies of the Drosophila proteome have used a variety of methods including matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry and ion-trap mass spectrometry. In addition, a separation step is often added to the analysis including reverse-phase liquid chromatography, ion-mobility spectrometry, or strong-cation-exchange chromatography. The number of genes, transcripts, and proteins that have been observed within the adult Drosophila are summarized in Figure 1-5A.

Initial proteomic methods have been used to understand the basic biology of Drosophila. Taraszka et al. were able to map the proteomes of three individual Drosophila heads identifying 197 proteins and finding at least 101 proteins in all three samples. While the other 96 proteins might not be expressed in all three fly samples, it
could also be that the flies were using different proteins for different functions at the time of sacrifice. Examples of these differences are shown in Figure 1-5B. More globally, differences have been observed in the *Drosophila* proteome lifespan. The fly proteome has been investigated over sixty days, at seven day increments. Approximately 1700 different proteins were identified and their changes in regulation compared between three different age groups: young (1-21 day old flies), middle (22-42 day old flies), and old (43-60 day old flies). Of these comparisons, a significant difference in protein regulation was observed for the young vs. middle groups. When the proteins experiencing an order of magnitude change or more in abundance were considered, 30 proteins were down-regulated while 12 proteins were up-regulated in the middle aged group. These proteins were found to be associated with metabolism, development, reproduction, or defense response.

Proteomic methods have yielded insight into Parkinson’s disease. Flies expressing either mutated A30P, mutated A53T, or normal human α-synuclein genes all display symptoms of Parkinson’s disease and have been investigated. These symptoms include decreased locomotor ability, formation of Lewy body-like inclusions in the brain, and degeneration of dopaminergic neurons with age. The symptoms are most severe for the flies with the A30P mutation, followed by the A53T mutated flies, and lastly the normal human α-synuclein mutated flies. All three of these mutated flies have had their proteomes compared to wild-type flies. Of note, the levels of 49 proteins in the A30P flies and 24 proteins in the A53T flies were significantly altered. Most of these proteins are associated with the actin cytoskeleton, mitochondria, and membrane. In the human α-synuclein mutant flies, only 12 protein changes were observed, mostly
related to metabolism and cellular signaling. These protein changes correlate with the severity of the Parkinson’s symptoms seen in the mutated flies and might lead to general insight about the protein alterations associated with this disease.

Complimentary to the genomic and proteomic work, *Drosophila* neuropeptides have also been investigated using MALDI-TOF mass spectrometry. Predel et al. characterized the adult fly peptidome using this technique and were able to identify 32 different neuropeptides in the *Drosophila* CNS. Not only did this reveal the occurrence of these neuropeptides, but it also depicted their morphological distribution. Recently, Kravitz and coworkers improved upon this method by combining both MALDI-TOF

**Figure 1-5** Mass spectrometric measurements of the *Drosophila* proteome. (A) Venn diagram of the known adult *Drosophila* genome (thin black circles and numbers), mRNA transcripts (thin grey circles and numbers), proteome (thick grey circles and bold numbers), and the overlap between mRNA transcripts and proteome (bold black italics numbers). Circle size corresponds to the number of known genes, transcripts, and proteins listed below the circle. (B) LC-IMS-MS analysis of three digested individual flies. Many of these features are common within all three individuals but some examples of the differences have been labeled. Circled features designate peptides found in all three individuals, boxes only two individuals, and triangles only one individual. (Reprinted with permission from the American Chemical Society).
mass spectrometry and electrospray ionization quadrupole time-of-flight (QTOF) mass spectrometry. Using the *Drosophila* galactosidase-4-upstream activating sequences (GAL4-UAS) gene targeting system, subsets of cells were genetically labeled to aid in sample preparation (explanation of the GAL4-UAS system is beyond the scope of this review, see reference. They were able to successfully identify 42 neuropeptides encoded by 18 different genes in adult *Drosophila* brain extract.

The larval *Drosophila* peptidome has also been investigated using both one- and two-dimensional (1D and 2D) capillary liquid chromatography (LC) followed by QTOF mass spectrometry. Baggerman et al. were able to identify 38 peptides using the 2D technique vs. 28 using the 1D technique. Their results demonstrate the increased efficiency of 2D LC/QTOF over its 1D counterpart.

Yew, Cody, and Kravitz applied atmospheric pressure ionization to studying cuticular pheromones by using direct analysis in real-time mass spectrometry. Since this technique can provide near instantaneous analysis of samples, pheromones could be chemically investigated over a long period of time from live, awake *Drosophila*. Flies were immobilized by a vacuum applied through a pipette tip and probed with a metal pin attached to a micromanipulator while still allowing the fly the ability to interact behaviorally with other flies. Pheromone levels were found to be increased in females vs. males, in females after courtship, and as one moves closer to the genitals of the male fly. While this work beautifully shows the spatial and temporal resolution that atmospheric pressure mass spectrometry can provide, it lacks the ability to measure analytes from inside of the fly.
Capillary Electrophoresis

CE separates ionic species according to their electrophoretic mobilities by applying a voltage over a narrow capillary filled with electrolytic solution. The small injection volumes associated with CE (nanoliters to femtoliters) make it an excellent method to study volume-limited samples such as *Drosophila*.

Moreover, CE has high resolving power due to its plug-like flow and minimal diffusion. Although CE uses electric fields to separate ions, neutral molecules can still be separated with CE by introduction of a surfactant to carry out micellar electrokinetic chromatography (MEKC). In MEKC, a surfactant, e.g. sodium dodecyl sulfate (SDS), is added to the running buffer at levels above the critical micelle concentration, resulting in the formation of micelles. The interaction of neutral molecules with the charged micelles results in retention and when this interaction is differential, separation results.

The Ewing laboratory has developed a procedure using MEKC to measure and quantify biogenic amines, their metabolites, and their precursors in *Drosophila*. End-column amperometry is used to selectively detect electroactive species providing a simple and sensitive detection method without the need for derivatization. Using MEKC coupled to electrochemical detection (MEKC-EC), they have investigated different anatomical regions of *Drosophila*, including whole body homogenates, whole head homogenates, and single head homogenates.
Investigating Different Parts of the Fly

Pioneering work by Ream et al. led to the development of a method that utilizes a N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES)/SDS buffer that is capable of separating seven biogenic amines, norepinephrine, epinephrine, octopamine, dopamine, tyramine, serotonin, and the dopamine precursor L-3,4-dihydroxyphenylalanine (L-DOPA), and this has been used to quantify four of these, dopamine, tyramine, serotonin, and L-DOPA, in the fly. To prepare homogenates for these studies, approximately 50-75 flies were flash frozen in liquid nitrogen and vortexed to separate the heads from the bodies. The intact fly heads and bodies were then collected in small tissue grinders and pulverized with 1 µL per head of 0.1 M perchloric acid, which helped to prevent enzymatic activity and air oxidation of electroactive species. These samples then were centrifuged, their supernatant collected, and then they were run through a 3-kDa filter. To compensate for any possible peak drifting, migration times from standards obtained both before and after the fly sample were used for peak identification as well as normalization to the migration time of an internal standard, here dihydroxybenzylamine (DHBA). The use of an internal standard also helps with any decay in coulometric efficiency that might be caused by fouling of the electrode from the complex biological sample.

Experiments were performed to explore this method’s ability to discriminate chemical differences within Drosophila and its genetic variants. To test for differences within the fly itself a collection of male heads (Figure 1-6A,B), male bodies made up of thoraces and abdomens (Figure 1-6C,D), and female heads (Figure 1-6E) were
homogenized and separated. Between the male (Figure 1-6A,B) and female (Figure 1-6E) head samples, no significant difference was noted in their separation profiles or amounts of basal biogenic amine content. Though two unidentified peaks at ~4 and 18 minutes were observed to decrease strikingly from the head to the body homogenates.

Figure 1-6 Enlargements of MEKC-EC electropherograms for a male head homogenate from 3-6 minutes (A) and 14-20 minutes (B) and male body homogenate from 2-5 minutes (C) and 12-18 minutes (D). These magnifications help to show the placement of the biogenic amines L-DOPA (1), internal standard DHBA (2), dopamine (3), tyramine (4), and serotonin (5). The asterisk marks an unknown peak consistency observed in both homogenates. (E) Comparison of female Canton-S (lower) and transgenic (upper) head homogenates identified with the same numbers as A-D. An enlargement of the transgenic homogenate is shown in the upper right hand corner to stress the tyramine and serotonin peaks. (Reprinted with permission from the American Chemical Society).
A higher abundance of dopamine in samples from the body was observed when compared to the head only samples. This may be a consequence of dopamine being a main contributor to the sclerotization (hardening) of the exterior cuticle of the fly body. In contrast to the dopamine levels, the levels of L-DOPA remained unaltered between the two fly preparations. Furthermore, the levels of serotonin and tyramine were found to be higher in the head with tyramine levels close to the limit of detection in the body. A transgenically modified fly with functionally ablated amino acid decarboxylase neurons was also compared to the wildtype strain (Figure 1-6E). As predicted, biogenic amines utilizing the amino acid decarboxylase enzyme, serotonin and dopamine, were considerably reduced in these flies. Serotonin was reduced by over ten-fold, while dopamine was not even detectable in the transgenic flies. However, tyramine levels stayed the same indicating this observation reflects a change in the biology of the fly and not an aberration in the system.

**Optimization of Metabolite Separations for Fly Samples**

The MEKC-EC separation with the TES/SDS buffer is a nice example of the hydrophobic elution order in MEKC as analytes interact with the pseudostationary phase (Figure 1-7A). In free-zone CE, species elute from the column according to their electrophoretic mobilities adjusted by electroosmotic flow, cations followed by neutral species and then anions. This is not the case for MEKC. The lone net neutral (zwitterionic) species, l-DOPA, eluted first from the capillary, while the anionic species, homovanillic acid, 5-hydroxyindoleacetic acid, and 3,4-dihydroxyphenylacetic acid,
migrated from the capillary clustered together with cationic analytes species eluting before and after them owing to the two separation mechanisms. In addition, larger species, in this separation the three tryptophan-derived analytes, have been shown to interact more with the micelle and thus elute later from the column. Of the N-acetylmetabolites, N-acetyloctopamine, N-acetyldopamine, and N-acetylserotonin, N-acetylserotonin was the largest and consequently eluted last. Similarly, serotonin is the last compound to elute in the entire separation.

![Figure 1-7](image-url)

**Figure 1-7** Standard separations of 100 µM samples for 14 biogenic amines in 10 mM TES/30 mM SDS/2% 1-propanol pH=7.09 buffer (A) and 14 biogenic amines in 25 mM borate/50 mM SDS/2% 1-propanol pH=9.50 buffer (B). Peaks are identified as follows: L-DOPA (1), N-acetyloctopamine (2), N-acetyldopamine (3), N-acetylserotonin (4), homovanillic acid (5), 5-hydroxyindoleacetic acid (6), 3,4-dihydroxyphenylacetic acid (7), norepinephrine (8), epinephrine (9), octopamine (10), dopamine (11), tyramine (12), 3-methoxytyramine (13), serotonin (14), and internal standard, catechol. (Reprinted with permission from the American Chemical Society).
While the separation with the TES/SDS buffer, as described by Ream et al, can be used to quantify and identify biogenic amines throughout the fly, only eleven of the fourteen analytes were resolvable. To improve the resolution of the separation of biogenic amines, the method was updated by use of a 25 mM borate/50 mM SDS/2% 1-propanol pH=9.50 buffer to resolve all the analytes. Borate (at basic pH) forms a complex with analytes possessing vicinal hydroxyl groups, imparting a negative charge to the complex and changing both the electrophoretic mobility of the analyte and its interaction with the micellar pseudo stationary phase. When applied to these 14 biogenic amines and optimized with attention to the high sensitivity to pH for this reaction, the borate buffer complexes with dopamine, norepinephrine, epinephrine, N-acetyldopamine, L-DOPA, and 3,4-dihydroxyphenylacetic acid to yield a fully resolved separation of all 14 analytes (Figure 1-7B). Not surprisingly comparing the separations using TES/SDS and borate/SDS buffers, it was these complexed moieties that had the largest changes in their elution time. The elution times for dopamine, epinephrine, and norepinephrine, now all net neutral, moved from the end to the beginning of the separation. The peaks for L-DOPA and DOPAC, now more negatively charged, moved, respectively, from the beginning to the middle and from the middle to the end of the separation.

Seven of these biogenic amines were found in homogenized samples of *Drosophila* heads. Previously detected dopamine, tyramine, and L-DOPA were seen in addition to octopamine, N-acetyldopamine, N-acetyloctopamine, and N-acetylserotonin. However, serotonin was not observed in these samples, possibly due to its shift in elution time to very late in the separation owing to prolonged interactions with the SDS micelles.
Using the borate/SDS buffer and MEKC-EC, separations of amines in wild-type *Drosophila* were compared to those from a mutant form of the fly, *inactive* (*iav*), which expresses lower levels of octopamine and tyramine. As expected, the amounts of N-acetyloctopamine, tyramine, and octopamine were reduced in the mutant form vs. the wild-type flies, with tyramine being present at levels below the limit of detection in the mutant flies.

*Separation of Biogenic Amines in the Head vs. Brain*

Use of small sample volumes with CE allows the study of a population of flies and the variability within individual fly heads. To do this, a special miniature tissue homogenizer was fabricated by pulling ~1 mm glass capillaries to form the homogenizing rod and Pasteur pipettes to form the homogenizing vessel with a micropipette puller. Individual fly heads can be collected in the vessel, homogenized with 0.1 M perchloric acid solution, sonicated, and injected directly from the vessel to the CE system. Using 1 µL of perchloric acid in the samples and the original 10 mM TES/30 mM SDS/2% 1-propanol pH=7.09 separation buffer to ensure serotonin detection, separation profiles of the single heads matched the previously reported population studies. By reducing the amount of this homogenizing solution to 750, 500, 250, and 100 nL, Powel et al showed that peak intensities can be enhanced. As the volume used was decreased, the overall profile of the fly separation remained rather consistent with increased resolution for a few
individual analytes, such as N-acetylserotonin and serotonin. The use of more concentrated samples resulted in increased peak intensities, but care must be taken to avoid overload of the electrochemical detector, which might cause a decrease in separation efficiency. Since a volume of 250 nL balances these two competing factors and gives more reproducible separations, it was used to investigate variances between single flies. Three individual heads were analyzed and compared. This procedure resulted in reproducible identification of nine neurochemicals (Figure 1-8),\textsuperscript{60} including N-acetyltyramine which was absent from the original population studies.

![Figure 1-8](image)

**Figure 1-8** (A) MEKC-CE separation of homogenate from a homogenized *Drosophila* single head with TES/SDS buffer. (B)-(C) Enlargement of the electropherogram emphasizes peaks L-DOPA (1), N-acetyl octopamine (2), N-acetyl dopamine (3), N-acetyl tyramine (4), N-acetyl serotonin (5), octopamine (6), internal standard DHBA. (Reprinted with permission from the American Chemical Society).

**Thesis Overview**

Many separation-based techniques have been developed and subsequently used to measure biogenic amines, their precursors, and their metabolites in various mammalian model systems such as rats, mice, and primates, but until recently few have utilized the potential offered by a model system such as *Drosophila melanogaster*. Not only do
Drosophila reproduce relatively quickly and are small enough where hundreds can be kept in a small cabinet, they offer one of the most powerful genetic tools available to a researcher. The work presented in this thesis encompasses both CE method development and application of CE instrumentation to studies using Drosophila.

An updated set of optimized separation conditions using CE-EC for the analysis of biogenic amines, their precursors, and their metabolites in homogenate fruit-fly populations is the objective of the work presented in Chapter 2. These experiments were aimed to increase the number of possible analytes that can be analyzed, especially the alcoholic metabolites salsolinol and norsalsolinol, by precisely controlling the pH of the separation conditions. Chapters 3 and 4 also expand upon using CE-EC to study biogenic amines in the fruit fly by investigating the use of dissection as a homogenization technique. Chapter 3 discusses the use of dissections as a sample clean-up mechanism to remove the large electrochemical responses present in the fly eyes. These substances coelute with the biogenic amines and make it difficult to separate and determine the substances in the brain alone. Different genetic variants have been investigated to determine what differences are present and ultimately to allow for the analysis of single fly brains and even specific sections of the fly brain. Chapter 4 takes a further step to analyze dissected regions of the fly brain, through the process of freeze-drying, to increase the speed of the dissection process for the analysis of fly populations.

The focus of the analytical method was changed somewhat from CE-EC to CE-MS in Chapter 5. Here the discussion concentrates more on expanding the detectable number of neuromodulators by optimizing a separation method that is suitable to use with CE-MS. Non-electroactive neurotransmitters glycine, glutamate, and GABA have been
investigated in addition to the biogenic amines. The method was then applied to detect the oral administration of methylphenidate.

References

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Chapter 2

Determination of Salsolinol, Norsalsolinol, and Twenty-one Biogenic Amines Using Micellar Electrokinetic Capillary Chromatography – Electrochemical Detection

Introduction

Biogenic amines, such as catecholamines (dopamine, norepinephrine, octopamine, etc) and the indoleamine serotonin, serve as neurotransmitters and are widely distributed within both the central and peripheral nervous systems. They have been implicated to play important roles in a variety of physiological processes from learning and memory, emotion, motion, sleep, and appetite as well as disorders such as depression, Parkinson’s Disease, and Huntington’s Disease. Precursors and metabolites of biogenic amines can also play important roles within neuronal pathways helping to modulate the overall activity of the brain and can be used as biomarkers for these disease states.\textsuperscript{1, 2} As such, it is desirable to develop new methodologies to identify and quantify not only neurotransmitters within the brain but their related presynaptic and metabolic compounds.

The dopamine-derived tetrahydroisoquinolines, salsolinol and norsalsolinol, are putative metabolic neuromodulators that have been found to be present in both rat\textsuperscript{3, 4} and human\textsuperscript{5, 6} brains. These compounds modulate dopaminergic transmission and have been shown to be associated with neurotoxicity within cells and diseases such as alcoholism and Parkinsonism. Salsolinol can be formed either by condensation of dopamine with pyruvate followed by oxidative decarboxylation and reduction (Figure 2-1A) or enzymatic-condensation of ethanol to acetylaldehyde with dopamine by the Pictet-Spengler mechanism (Figure 2-1B).\textsuperscript{7-9} Due to its
direct chemical link to ethanol, studies on salsolinol have been in relation to alcohol use and abuse. Salsolinol has been found to be elevated in alcoholic patients\textsuperscript{10, 11} and induces reinforcement in self-administration experiments\textsuperscript{12}. Norsalsolinol can be synthesized in vivo nonenzymatically by condensation of dopamine with formaldehyde, the oxidized metabolite of methanol (Figure 2-1B). It has been linked primarily with neurotoxicity, especially within Parkinson’s Disease\textsuperscript{13}, but may be of interest in alcohol withdrawal due to increased methanol levels\textsuperscript{9}.

Figure 2-1: Biosynthesis and structures of the dopamine derived isoquinolines, salsolinol and norsalsolinol. (A) One pathway through which salsolinol can be formed in vivo involves dopamine condensing with pyruvate, removal of the carboxylate group, and reduction. (B) Salsolinol and norsalsolinol can both be formed by a Pictet-Spengler reaction. Initially an alcohol, either methanol or ethanol, is oxidized by alcohol dehydrogenase (ADH) to its respective aldehyde. That aldehyde then reacts with dopamine to form norsalsolinol or salsolinol.
The fruit fly *Drosophila melanogaster* is a highly investigated model system for neuronal processes. In contrast to a rat or mouse model, *Drosophila* are smaller in size and have shorter life cycles making them easier to keep in larger quantities, while their fewer genetic functional redundancies and fully mapped out genome\textsuperscript{14} have produced a plethora of genetic mutants available for study. Furthermore, many similarities exist between the genetics and function of *Drosophila* and mammals. Both systems contain similar molecular and cellular components, particularly neuromodulators such as dopamine, and physiological systems involving basic cellular function and signal transduction pathways.\textsuperscript{15-18} Several separations-based techniques have been employed to study neuromodulators in *Drosophila*, including HPLC with EC detection,\textsuperscript{19-23} GC-MS,\textsuperscript{24} and MEKC-EC.\textsuperscript{25-27} Whereas both HPLC and GC have been shown to be viable methods to quantify the neurochemicals present in *Drosophila*, use of these techniques only allows a select few neuromessengers to be determined, usually the main neurotransmitters. Conversely, the separation and identification of fourteen biogenic-amine modulators and metabolites in the fly has been demonstrated with MEKC-EC.\textsuperscript{26}

While many studies have been performed to help better understand the precise roles salsolinol, norsalsolinol, and other neuronal metabolites play within the body, quantification of putative neuromessengers challenges our current analytical techniques due to small sample volumes and interferences from the complex biological matrix. A small-volume separation with high selectivity would be a valuable addition to the repertoire of methods available here.

MEKC is a mode of capillary electrophoresis where surfactant exceeding the critical micelle concentration is added to the running buffer. The system benefits from fast separation times, high-resolution capabilities, and low mass detection limits from the capillary electrophoresis system, and the extra surfactant generates a second degree of separation that
affords the separation based on hydrophobicity of the analyte. Moreover when combining this system with amperometric EC detection, one gains a highly sensitive and selective system that does not necessitate sample derivatization and addresses the aforementioned complexities of measurements in biological samples.

In this chapter, previous the work has been built upon to develop a separation scheme to further investigate monoamine modulators, their metabolites, and related molecules (referred to as “metabolites” here) present in the Drosophila central nervous system. A sequential, more precise optimization of the borate/SDS micelle buffer using \([\text{OH}^-]\) is presented. Not only does this method improve upon resolution of the fourteen previously reported neurometabolites using borate complexation, but also nine new metabolites are added to the separation, bringing the separation total to twenty-three compounds plus the internal standard. Standard additions were performed in order to quantify metabolites in Canton-S wild-type Drosophila to verify the method in biological samples and to show the presence of salsolinol and norsalsolinol in the fly.

**Experimental Section**

**Reagents**

Sodium hydroxide and DL-tyrosine were obtained from Fluka (Buchs, Switzerland), perchloric acid from Riedel-de Haën (Morristown, NJ, USA) and norsalsolinol from Acros organics (Geel, Belgium). N-acetylamine dopamine, N-acetylamine octopamine, and N-acetylamine tyramine were obtained from the The National Institutes of Mental Health (NIMH) chemical synthesis and drug supply program (Research Triangle Park, NC, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Separations were
performed in 25 mM borate buffer containing 50 mM SDS and 2% 1-propanol with various concentrations of NaOH. All standards were prepared as 10 mM stock solutions in 0.1 M perchloric acid and were diluted to the desired concentration with additional 0.1 M perchloric acid.

**Drosophila Strains**

Canton-S wild-type *Drosophila melanogaster* strains were obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN, USA) and were maintained in the laboratory. Flies were cultured on standard potato meal/agar medium and collected for homogenization 4 and 5 days after emerging from pupal cages.

**Homogenate Preparation**

Flies were prepared for CE experiments based on a protocol previously described. Flies were collected in 15-mL centrifuge tubes, plunged into liquid nitrogen, and vortexed for 60 s in order to separate the heads from the body. Approximately 100 heads were counted, collected, and homogenized in 0.1 M perchloric acid/100 µM catecholamine (internal standard) at a volume of 0.5 µL/head. The homogenized heads were centrifuged using an Eppendorf centrifuge with a fixed angle rotor (Brinkman Instruments, Westbury, NY, USA) and spun at 14,000 rpm for 5 min at 0 °C. The supernatant was collected and centrifuged (2 h, 14,000 rpm, 0 °C) through a 3-kDa centrifuge filter (VWR International, LLC, West Chester, PA, USA). The filtrates were dispensed in aliquots of 4.5 µL and stored in the freezer (-80 °C) until use.
**Instrumentation and Analysis**

The CE system with end-column amperometric detection utilized in this study was built in-house and has been described previously. Briefly, 45-50 cm of fused-silica capillary with an outer diameter of 151 µm and inner diameter of 14 µm (Polymicro Technologies, Phoenix, AZ) was used for the separations. Amperometric EC detection was carried out with a two-electrode format in which a 5-µm carbon fiber microelectrode was held +0.75 V versus a Ag/AgCl reference electrode (Harvard Apparatus, Holliston, MA, USA). The current was measured using a Keithly model 427 current amplifier (Cleveland, OH, USA) and recorded at 10 Hz using a LabView 8.0 (National Instruments, Austin, TX, USA) interface, written in-house. Microsoft Excel (Redmond, WA, USA) was used to generate the electropherograms and statistics. Data analysis was performed using Peakfit Separation and Analysis Software v4.11 (SPSS Inc., Chicago, IL, USA).

**Procedures**

Buffer solutions were filtered with a 0.2 µm nylon filter (Alltech, Deerfield, IL) prior to use. Capillaries were filled with separation buffer using a stainless steel reservoir with applied N₂ pressure (3.5 MPa) and washed between changes in buffer with deionized water (18.0 MΩ) for ~15 min. Injections were then performed electrokinetically at 5 kV for 5 s to extract ~1 nL of homogenate from the homogenate. To enhance microelectrode placement, the capillary inner diameter was enlarged via HF etching as previously described. Approximately 2 mm of the polyimide coating was burned off from the capillary to expose the fused silica. The exposed portion of the capillary then was placed in HF for 15 min with a pressure of 3.5 MPa (N₂)
through the capillary. Subsequently, the same segment of capillary was placed in a sodium bicarbonate solution to neutralize the acid and then washed with water to remove any excess salt. Typically, the same capillary was employed for any single set of experiments such as all the samples in the set of standard additions or calibration curve.

The buffers with small pH changes to optimize separations were made as follows. Five liters of 25 mM borax/50 mM SDS/2% 1-propanol were made and divided into equal 0.250 L portions. A small amount of OH− was added to each portion sequentially increasing the concentration from 6.96 mM to 44.79 mM. The pH of the solution was then calculated based on the buffer present and amount of hydroxide added.

Safety Considerations

An in-house-built safety interlock box was utilized to protect the user from high voltage. Since HF could cause severe burns, it was used with extreme care in a fume hood with proper precautions taken.

Results and Discussion

Optimization of Borate/SDS Buffer With [OH−]

Twenty-three electroactive biogenic amines and metabolites were selected for separation optimization and to better understand the possible roles they might play in the neurophysiology of Drosophila. Previous work applied separation buffers with 25 mM sodium tetraborate, 50 mM SDS, and 2% 1-propanol at pH 9.50 to investigate neurotransmitters and metabolites using
MEKC-EC. These included L-DOPA, norepinephrine, epinephrine, octopamine, dopamine, tyramine, serotonin, DOPAC, 5-hydroxyindoleacetic acid, 3-methoxytyramine, homovanillic acid, N-acetyldopamine, N-acetylserotonin, N-acetyloctopamine, and N-acetyltyramine. Borate complexation with vicinyl hydroxyl groups helped to resolve a larger number of analytes. This adds a net negative charge to the borate-analyte complex and alters the overall migration behavior of the analyte in the electric field and its interaction with the SDS. Borate forms complexes with nine analytes in the separations. Six compounds, dopamine, norepinephrine, epinephrine, N-acetyldopamine, L-DOPA, and DOPAC have been used previously, while three salsolinol, norsalsolinol, and 3,4-dihydroxymandelic acid, are new.

Previously, borate concentration, SDS concentration, and pH were investigated to optimize the separation conditions of this running buffer. Borate concentration had little effect on resolution, while increased SDS concentration increased resolution. Altering the pH of the separation buffer caused the resolution, peak profiles, elution time reproducibility, and elution times to vary. To prevent upsetting the sensitive balance previously obtained, initial experiments carried out here used the same pH and increased the concentrations of the SDS in the running buffer attempting to fine-tune the resolution of all twenty-three analytes. This was, however, not enough to resolve these analytes, particularly dopamine, salsolinol, and norsalsolinol, which elute first from the capillary. As previously discussed, salsolinol and norsalsolinol are structurally similar metabolites of dopamine (Figure 2-1). As salsolinol and norsalsolinol are metabolites of dopamine and they differ from one another by only a methyl group, it is not surprising that these analytes have similar mobilities. A more powerful method of optimizing the separation conditions was investigated to resolve these three analytes.
As many of the biogenic amines of interest have primary amines, their pKa’s are between 9 and 10; it was surmised that small changes in the running buffer pH of 9.50 would have dramatic effects on the separation selectivity and resolution of specific species. The constituents of the buffer made pH changes of 0.01 difficult to measure, hence, a method where the pH was back calculated from the total amount of base added to the buffer and optimized relative to the separation of these analytes was used. Separations of dopamine, salsolinol, norsalsolinol, N-

![Figure 2-2](image)

**Figure 2-2** Standard separations of 100 µM (1) dopamine, (2) salsolinol, (3) norsalsolinol, (5) N-acetylocopamine, and (6) octopamine with increasing pH (added aliquots of [OH⁻]) to optimize separation resolution between analytes. Amounts of [OH⁻] aliquots correspond to calculated pH values of (a) 9.26, (b) 9.30, (c) 9.51, (d) 9.61, (e) 9.70, (f) 10.15, and (g) 10.40. All separations were run sequentially on the same 45-cm piece of capillary using the same electrode at 15 kV with a 15-min wash of deionized water between buffer changes.
acetyloctopamine, and octopamine were then performed with incremental amounts of hydroxide ion added to the running buffer. The capillary was washed with 18 MΩ H₂O for 15 min between each run to ensure removal of the old running buffer. Representative electropherograms of these separations are shown in Figure 2-2.

The values for separation resolution for the DA-salsolinol and salsolinol-norsalsolinol pairs following different additions of hydroxide (noted as calculated changes in pH) are shown in Figure 2-3A. Initially, the resolution of DA and salsolinol increased with increased pH going from an R of <0.5 to a maximum of 3.2 from pH 9.26 to 10.01. At even higher pH, the resolution then dropped dramatically, as all the available boric acid was converted to its singly deprotonated species. Conversely, salsolinol and norsalsolinol coeluted until pH 9.53. At higher pH, the resolution of these two species increased, peaked at R = 1.2, and dropped for the highest tested pH values. The separation resolution of N-acetyloctopamine with its two closest peaks, norsalsolinol and octopamine, were also calculated (Figure 2-3B). At the lowest and highest pH values tested, N-acetyloctopamine was highly resolved from octopamine (R = 12-16) but not as well resolved from norsalsolinol (R < 2). As the pH shifted away from the most extreme values, the norsalsolinol-N-acetyloctopamine resolution increased and N-acetyloctopamine-octopamine resolution decreased. These resolution values reached both minimum and maximum values in the narrow range between pH 9.5 and 9.8 for the run buffer.
The pH = 9.66 value was chosen as optimal. Under this condition, DA was resolvable from salsolinol without being so close to the pH values where a very small change would lead to a large change in separation resolution. Similar logic applies to the ability to resolve salsolinol from norsalsolinol and N-acetyloctopamine from norsalsolinol and octopamine. This scheme produced a separation with sharper peak shapes and fewer impurity peaks, and helped to keep analysis times less than 30 min, all of which have impact on quantification.

**Figure 2-3** Resolution plots for the peaks from Figure 2-2. (A) Resolution for dopamine-salsolinol (circles) and salsolinol-norsalsolinol (diamonds) versus the calculated pH of the OH⁻ aliquots added. Values generally increased with increasing pH until a pH of 10.01 where the resolution dropped sharply. (B) Resolution calculations for N-acetyloctopamine and its two closest peaks, norsalsolinol (diamonds) and octopamine (circles), versus the calculated pH for OH⁻ aliquots added.
The optimized separation of twenty-three amines and metabolites is shown in Figure 2-4. Peak identities were elucidated by matching their elution times with that of each individual biogenic amine or metabolite measured under the same buffer conditions. The same capillary and electrode were used for all consecutive separations. Under these conditions, nine new analytes were separated and the elution order for the compounds examined previously changed elution order. The elution positions of two previously reported analytes, norepinephrine and homovanillic acid, changed elution order. Salsolinol and norsalsolinol eluted from the capillary second and third, between dopamine and epinephrine, followed by N-acetyloctopamine, octopamine, N-acetylserotonin, and norepinephrine. Although slight, the change in pH used was enough to move norepinephrine by two places in the elution order, whereas the newly added metabolite N-acetylttyramine eluted before N-acetyldopamine, 5-hydroxyindoleacetic acid, vanillylmandelic acid, and L-DOPA. The internal standard catechol switched positions with homovanillic acid from the original elution order and these were followed by ascorbic acid, 3,4-dihydroxymandelic acid, and p-hydroxymandelic acid, tyramine, 3-methoxytyramine, tyrosine, serotonin, and DOPAC.

The optimized separation conditions make possible the identification of previously unstudied compounds salsolinol, norsalsolinol, N-acetylttyramine, vanillylmandelic acid, 3,4-dihydroxymandelic acid, p-hydroxymandelic acid, ascorbic acid, guanine, and tyrosine. Salsolinol and norsalsolinol are metabolites of dopamine associated with alcohol use and abuse, whereas N-acetylttyramine is the major metabolite of tyramine and a putative neuromodulator in the Drosophila central nervous system. Peaks corresponding to guanine (peak 19 in Figure 2-4), tyrosine (peak 22 in Figure 2-4), and DOPAC (peak 24 in Figure 2-4) are significantly smaller
than those of the other analytes. The small peak size for guanine might result from poor solubility of this compound. Although guanine has been found to be soluble in acidic solutions\textsuperscript{29} and standard solutions were made with 0.1M HClO$_4$, low solubility in the basic running buffer might result in stronger interactions with the SDS micelles. Similarly, the strongly-polar amino-acid group of tyrosine might lead to increased interactions with the SDS micelles and thus explain its low, broad peak. The peak for DOPAC is typically low in amplitude and broad in these separations as it elutes late and the highly anionic nature of the borate complex leads to poor electron transfer kinetics at carbon electrode such as those used here for detection. Even though some of these analytes have been studied using HPLC,\textsuperscript{20, 22, 23, 30} these twenty-three biogenic amines have not been resolved in a single electrophoretic separation previously.

To demonstrate the quantitative ability of this method, calibration curves of standards (concentrations from 1 µM to 40 µM) were plotted for all twenty-three analytes (Table 2-1).
Each of the sets of standards were run sequentially three times using the same electrode and capillary on the same day, but a fresh electrode, capillary, and running buffer were prepared for each new set of standards. The use of an internal standard catechol compensates for variation due to sample handling, injection efficiency, and fouling of the working electrode. The resulting calibration plots were linear with correlation coefficients of 0.97 or greater for fifteen of the analytes, including the first 14 compounds eluting from the column in the separation. Two more compounds, ascorbic acid and 3,4-dihydroxymandelic acid, had correlation coefficients just under 0.97 at 0.92 and 0.95, respectively. The last six compounds eluting from the capillary (guanine, tyramine, 3-methoxytyramine, tyrosine, serotonin, and DOPAC) had correlation coefficients less than 0.90. This is not entirely unexpected as the increased time in the capillary might indicate stronger interaction with the SDS micelles, thus creating additional band broadening.
Table 2-1  Regression parameters for three independent calibrations of twenty-three biogenic amines and metabolites in the optimized borate/SDS buffer system. For intercepts where the variance was larger than the value calculated, values are reported as zero.

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Slope ± Standard Deviation</th>
<th>Intercept ± Standard Deviation</th>
<th>Correlation Coefficient (r)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>2.07 ± 0.094</td>
<td>-1E-05 ± 1.5E-05</td>
<td>0.98</td>
</tr>
<tr>
<td>Salsolinol</td>
<td>1.08 ± 0.067</td>
<td>0 ± 1.1E-05</td>
<td>0.97</td>
</tr>
<tr>
<td>Norsalsolinol</td>
<td>1.7 ± 0.11</td>
<td>0 ± 1.7E-05</td>
<td>0.97</td>
</tr>
<tr>
<td>Epinephrine</td>
<td>2.0 ± 0.11</td>
<td>-2E-05 ± 1.7E-05</td>
<td>0.98</td>
</tr>
<tr>
<td>N-acetyloctopamine</td>
<td>0.81 ± 0.042</td>
<td>0 ± 6.8E-06</td>
<td>0.98</td>
</tr>
<tr>
<td>Octopamine</td>
<td>1.47 ± 0.061</td>
<td>6E-06 ± 9.9E-06</td>
<td>0.99</td>
</tr>
<tr>
<td>N-acetylserotonin</td>
<td>1.76 ± 0.078</td>
<td>-4E-05 ± 1.3E-05</td>
<td>0.99</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>2.24 ± 0.077</td>
<td>-1E-05 ± 1.3E-05</td>
<td>0.99</td>
</tr>
<tr>
<td>N-acetyltlyramine</td>
<td>0.77 ± 0.035</td>
<td>6E-06 ± 5.8E-06</td>
<td>0.98</td>
</tr>
<tr>
<td>N-acetyldopamine</td>
<td>0.70 ± 0.026</td>
<td>0 ± 4.2E-06</td>
<td>0.99</td>
</tr>
<tr>
<td>5-hydroxyindole acetic acid</td>
<td>0.61 ± 0.038</td>
<td>0 ± 6.2E-06</td>
<td>0.97</td>
</tr>
<tr>
<td>Vanillylmandelic acid</td>
<td>1.17 ± 0.056</td>
<td>0 ± 9.2E-06</td>
<td>0.98</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>2.2 ± 0.11</td>
<td>-2E-05 ± 1.7E-05</td>
<td>0.98</td>
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<tr>
<td>Homovanilic acid</td>
<td>0.67 ± 0.035</td>
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</tr>
<tr>
<td>Ascorbic Acid</td>
<td>0.54 ± 0.057</td>
<td>1.6E-05 ± 9.3E-06</td>
<td>0.92</td>
</tr>
<tr>
<td>3,4-dihydroxymandelic acid</td>
<td>0.50 ± 0.041</td>
<td>6E-06 ± 6.6E-06</td>
<td>0.95</td>
</tr>
<tr>
<td>p-Hydroxymandelic acid</td>
<td>1.84 ± 0.084</td>
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<td>0.98</td>
</tr>
<tr>
<td>Guanine</td>
<td>0.33 ± 0.056</td>
<td>1.1E-05 ± 9.1E-06</td>
<td>0.82</td>
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<tr>
<td>Tyramine</td>
<td>2.0 ± 0.43</td>
<td>3E-05 ± 7.0E-05</td>
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</tr>
<tr>
<td>3-methoxytyramine</td>
<td>0.21 ± 0.030</td>
<td>1.0E-05 ± 4.9E-06</td>
<td>0.87</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.11 ± 0.055</td>
<td>0 ± 1.3E-05</td>
<td>0.65</td>
</tr>
<tr>
<td>Serotonin</td>
<td>0.20 ± 0.036</td>
<td>1.1E-05 ± 5.9E-06</td>
<td>0.81</td>
</tr>
<tr>
<td>DOPAC</td>
<td>0.07 ± 0.035</td>
<td>4E-06 ± 3.5E-06</td>
<td>0.55</td>
</tr>
</tbody>
</table>
Identification of Salsolinol and Norsalsolinol in Wild-Type *Drosophila* Head Homogenates

To demonstrate the applicability of using this method in bioanalysis, dopamine, salsolinol, norsalsolinol, N-acetyloctopamine, octopamine, and N-acetyldopamine have been separated in head homogenates of the wild-type *Drosophila* strain Canton-S have been separated and quantified. Standard additions of 40, 20, 10, 5, 1 and 0.5 µM of these substances were used. Previous reports utilized comparisons of standard separations run before and after the homogenate separation, but standard addition was chosen for this analysis to reduce any possible matrix effects and to aid in peak identification within the multitude of unidentified electroactive species present in a homogenate sample.

To prevent dilution of the head homogenates as much as possible and ensure consistency between samples, head homogenates were split into 4.5 µL aliquots and spiked with 0.5 µL of standard spiking solution with concentration corresponding to the correct final concentration of standard in the sample. Although no statistical difference in basal monoamine levels has been observed in the fly heads between genders, gender differences to alcohol sensitivity have occurred. Therefore, only male flies were used in fly homogenates.

Figure 2-5A shows a sample electropherogram of a full separation of a *Drosophila* homogenate including the internal standard catechol. Homogenates were divided into six samples and spiked with standards. Enlargements of the first 400 s of a set of standard additions (Figure 2-5B) consistently identified peaks corresponding to dopamine, salsolinol, norsalsolinol, N-acetyloctopamine, octopamine, and N-acetyldopamine as the amount of the standard increase in the spiked samples. Mobilities from standard electropherograms were then compared to verify these analyte positions.
Figure 2-5 (A) MEKC-EC separation of a *Drosophila* male head homogenate highlighting the internal standard catechol (peak 13) and breadth of the separation. The five large peaks (signals greater than 100 pA) were unidentified. The peaks for all identified biogenic amines and metabolites were closer to the baseline and represented perhaps medium level substances. (B) Enlargements of electropherograms examining times from 400-800 s of the separation shown in (A) with standard additions of the known substances. Final concentrations of spiking standard solutions were (a) 0.5 µM, (b) 1 µM, (c) 5 µM, (d) 10 µM, (e) 20 µM, and (f) 40 µM for the six analytes. Numbered peaks correspond to (1) dopamine, (2) salsolinol, (3) norsalsolinol, (4) N-acetyloctopamine, (5) octopamine, and (6) N-acetyldopamine.
Standard additions were performed on three independent preparations of Canton-S head homogenates that have been analyzed by using this optimized borate/SDS MEKC-EC method, and these values are reported in Table 2-2. As reported in previous studies, some variability was present and expected between fly cultures possibly resulting from subtle differences in the environment or variability in sample homogenization efficiency, and sample handling. As before, the internal standard helped to compensate for variation due to sample handling, injection efficiency, and fouling of the working electrode, but not differences in the environment. Only the analysis of N-acetyldopamine proved to be difficult to complete (correlation coefficients for the three fly cultures ranged between 0.70 and 0.83) because of its proximity to several large unidentified electroactive peaks. The other analytes, dopamine, salsolinol, norsalsolinol, N-acetylloctopamine, and octopamine were identified and quantified consistently in all three cultures with correlation coefficients of 0.95 or greater. This optimized separation scheme has thus been used to measure previously reported as well as novel analytes in the fly head.

Table 2-2 Quantification of dopamine, salsolinol, norsalsolinol, N-acetylloctopamine, octopamine, and N-acetyldopamine using standard addition of three separate wild-type Drosophila head homogenates. Values reported for each analyte include; the calculated concentration of the analyte (C), correlation coefficient of the standard addition regression (r), and mean and standard error of the mean (SEM) for the three fly cultures.

<table>
<thead>
<tr>
<th>Fly culture</th>
<th>Dopamine</th>
<th>Salsolinol</th>
<th>Norsalsolinol</th>
<th>N-acetylloctopamine</th>
<th>Octopamine</th>
<th>N-acetyldopamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.8</td>
<td>2.08</td>
<td>2.0</td>
<td>5.3</td>
<td>12.6</td>
<td>16.5</td>
</tr>
<tr>
<td>2</td>
<td>1.5</td>
<td>2.21</td>
<td>2.3</td>
<td>4.8</td>
<td>10.9</td>
<td>18.1</td>
</tr>
<tr>
<td>3</td>
<td>1.1</td>
<td>2.23</td>
<td>0.8</td>
<td>4.2</td>
<td>9.2</td>
<td>19.8</td>
</tr>
<tr>
<td>Mean ± S.E.M.</td>
<td>1.5 ± 0.21</td>
<td>2.17 ± 0.048</td>
<td>1.7 ± 0.44</td>
<td>4.8 ± 0.32</td>
<td>10.9 ± 0.97</td>
<td>18.1 ± 0.95</td>
</tr>
</tbody>
</table>
Conclusions

An improved separation scheme has been developed for the analysis of biogenic amines and metabolites present in *Drosophila melanogaster* samples, which should also be applicable to other biological systems. The separation is extremely sensitive to small changes in pH, hence, optimization of the separation by careful addition of known amounts of OH⁻ proved more reliable than the more common adjustments using a pH meter. Under these conditions, twenty-four standards (twenty-three of potential interest and one internal standard) are resolved. These include key players that might play a role in alcohol tolerance and reward, such as dopamine, salsolinol, norsalsolinol, N-acetyloctopamine, and octopamine. These five biogenic amines and putative neuromodulators were identified and quantified in the *Drosophila* head using this methodology.
References


Chapter 3
Biogenic Amines in Microdissected Brain Regions of Drosophila melanogaster Measured with Micellar Electrokinetic Capillary Chromatography – Electrochemical Detection

Introduction

*Drosophila melanogaster* has been a broadly used model organism due to many of its inherent features. *Drosophila*’s small size (<0.5 cm) and short reproduction life cycle (12-14 days from conception to sexual maturity) have made it feasible to keep large quantities of genetically homogenous samples available to study. Fewer genes and fewer functional redundancies have facilitated the identification of individual genes\(^1,\)\(^2\) and ultimately produced a vast number of genetic mutants available to help investigate the physiological mechanisms underlying behavior.\(^3\) Pigmentation mutations result in easily identifiable phenotypes and are the oldest identified genetic mutation to be isolated in *Drosophila*, specifically the *white* mutant.\(^4\) In *white* mutants, the ATP-binding cassette (ABC) transporter, which transports guanine and tryptophan precursors to the red (drosoperterin) and brown (ommochrome) screening pigments into fly larvae eyes, is not properly expressed and thus prevents the formation of eye pigments.\(^5,\)\(^6\) As such they suffer from defective vision stemming from their inability to screen bright lights, difficulties in movement,\(^7,\)\(^8\) and deficiencies in learning.\(^9\) Care must be taken to compare them to their wild-type counterparts.
Although *Drosophila* has a less complex nature, due to its smaller genome and simpler nervous system of approximately 200,000 neurons, it can exhibit many of the same high-ordered behavioral and molecular functions found in vertebrates. Biogenic amines such as dopamine, serotonin, tyramine, and octopamine are known mediators of diverse physiological and behavioral functions within the fly. Despite the important role these molecules play within the nervous system, the underlying neuronal mechanisms are still not completely understood.

Traditionally, detection of biogenic amines within the *Drosophila* brain has been studied using immunohistochemical, histofluorescence, and pharmacological methods. These methods are invaluable tools for visualization of neurons containing biogenic amines; however, they are ineffective in quantifying them. More recently, small-volume methods have been developed for analysis in *Drosophila*. These include dynamic methods, especially voltammetry in *Drosophila* larvae and adult flies, and several small-volume separations-based techniques. Early separation experiments to quantify biogenic amines in *Drosophila* heads utilized gas GM-MS and HPLC with EC detection, but these studies utilized complex extraction and derivatization protocols or could only detect a few biogenic amines, mainly dopamine. Recent HPLC-EC methods have improved upon the separation methodology to increase the number of quantifiable biogenic amines and have used dissected fly brains to reduce the number of interfering electroactive compounds. MEKC is a mode of CE where surfactant exceeding the critical micelle concentration is present in the running buffer. The high-resolution capabilities and low mass detection limits of MEKC have been used to detect
biogenic amines and their modulators in both head homogenates and individual heads of the fly.\textsuperscript{41}

In this chapter, MEKC is used to investigate the levels of biogenic amines and their metabolites present in dissected whole brains and specific \textit{Drosophila} brain regions. The analytical challenge here has been to develop the protocols to look at even smaller tissues from a complex brain substrate. Furthermore, different fly strains, wild-type (Canton S), \textit{white} mutant, and GFP-TH mutant, have been used to investigate the effects fly-eye pigment has on these separations and analyze specific regions within the fly brain. Significant differences are observable between these different fly types and some brain regions.

**Experimental**

**Chemicals**

All chemicals were used as received and purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. Perchloric acid was obtained from Honeywell Riedel-de Haën (Seelze, Germany), while N-acetyldopamine, N-acetyloctopamine, and N-acetyltyramine were obtained from The National Institutes of Mental Health (NIMH) chemical synthesis and drug supply program (Research Triangle Park, NC, USA). Separations were performed in 25 mM borate buffer containing 50 mM SDS and 2% 1-propanol at pH = 9.66. All standards were prepared as 10 mM stock solutions in 0.1 M perchloric acid and were diluted to the desired concentration with additional 0.1 M perchloric acid.
**Drosophila Strains and Preparation**

Three strains of *Drosophila melanogaster*, wild-type Canton S, *white* mutant, and TH-GFP mutant, were used in this study. Canton S flies have red-pigmented eyes, while the *white* mutant strain contains a mutated copy of the *w* gene affording unpigmented eyes. TH-GFP flies are a transgenic strain that carry tyrosine hydroxylase (TH)-GAL4 and UAS-mCD::GFP (membrane tethered green fluorescent protein) which is used to visualize dopamine neurons. Images were acquired using a stereomicroscope (Olympus, SZX10, Hamburg, Germany) and an Olympus DP71 camera. All flies were cultured on standard potato meal/agar medium at 25°C and collected 4-5 days after emerging from pupal cages.

Two types of fly samples were prepared, head homogenates and dissected brains (see below). Collection of all fly samples began by briefly immobilizing male flies on ice for 10 s, dipping them in a bath of cold 95% ethanol for 30 s to remove their waxy coating, and placing them in a petri dish containing 0.1 M perchloric acid on ice. Fly heads were removed from their bodies using small forceps (Fine Science Tools, Heidelberg, Germany) under an Olympus SZ40 stereoscope.

Homogenates were prepared by collecting three heads and homogenizing in 1.0 µL of 0.1 M perchloric acid/100 µM catecholamine (internal standard). The homogenized heads were centrifuged using an Eppendorf centrifuge with a fixed angle rotor (Eppendorf, Hamburg, Germany) and spun at 14,000 rpm for 5 min at 0 °C. The supernatant was collected and centrifuged (2 h, 14,000 rpm, 0 °C) through a 3-kDa centrifuge filter (VWR International, LLC, West Chester, PA, USA). The filtrates were then immediately injected into the capillary.

Fly brains were dissected from their heads using small forceps under an Olympus SZ40 stereoscope. To dissect a fly, it was held at the belly facing up with one pair of forceps, while a second pair pinched at the neck to separate the head from the body. The proboscis was removed.
by pulling it down and away from the head to create a cavity in the lower region of the head. Holding onto each side of the cavity, forceps were gently pulled away from one another until the cuticle was broken into two pieces making sure the brain stayed intact. The brain was removed from the pieces of cuticle, eliminating any remnants of the air sacs, compound eye, or cuticle debris. Brain region samples were collected by further dissecting a TH-GFP brain under fluorescent light to visualize the central brain, optic lobes, and PPM1 regions. All individual dissections were performed in less than 5 min to prevent the fly from being dead for too long as analyte levels may change due to the stress placed upon the fly. A discussion of fly dissections has been described in Nature Protocols.42

Dissected brains or brain regions were collected in 200-µL PCR tubes (VWR International, West Chester, PA, USA) with 1.0 µL of 0.1 M perchloric acid/25-100 µM catecholamine (internal standard) and centrifuged (VWR International, Galaxy Mini, West Chester, PA, USA) at 6000 RPM for 30 s. The heads were then ultrasonicated (Sonics & Materials, Inc, Satigny, Switzerland) with a 2 mm stepped microtip at 100% amplitude for 30 s in a cold-water bath. The sample was centrifuged for 30 s for collection and promptly injected onto the capillary.

Separation Instrumentation and Analysis

The CE system with end-column amperometric detection utilized in this study was built in-house and has been described previously.43, 44 A 45-cm length of fused-silica capillary with an outer diameter of 151 µm and inner diameter of 14 µm (Polymicro Technologies, Phoenix, AZ, USA) was used for the separations. Amperometric EC detection was carried out in a two-electrode format in which a 5-µm carbon fiber microelectrode was held +0.75 V versus a Ag/AgCl reference electrode (Harvard Apparatus, Holliston, MA, USA). Carbon-fiber
microelectrodes were fabricated by aspirating a 5 µm diameter carbon fiber (Amoco, Greenville, SC, USA) into a borosilicate glass capillary (B120-69-10, Sutter Instruments, Novato, CA, USA). The glass capillary was then pulled to a narrow tip with a glass-capillary puller (Narishige, PE-21, Tokyo, Japan) and epoxy (Epo-Tek, Epoxy Technology, Billerica, MA, USA) was placed at the carbon-fiber/glass junction and allowed to cure. The carbon fiber was cut to a length of 400-500 µm from the glass junction to form a cylindrical electrode. Electrical contact was made by back-filling the capillary with 3.0 M KCl and inserting a Ni:Cr wire. Current was measured using a Keithly model 428 current amplifier (Cleveland, OH, USA) and recorded at 10 Hz using a LabView 8.0 (National Instruments, Austin, TX, USA) interface, written in-house. Microsoft Excel (Redmond, WA, USA) was used to generate the electropherograms and statistics. Data analysis was performed using Peakfit Separation and Analysis Software v4.11 (SPSS Inc., Chicago, IL, USA).

Buffer solutions were filtered through a 0.2-µm nylon filter (Alltech, Deerfield, IL, USA) prior to use. Capillaries were filled with separation buffer using a stainless steel reservoir with applied N₂ pressure (3.5 MPa) and washed between changes in buffer with deionized water (18.0 MΩ) at 15 kV for ~15 min. Injections were then performed electrokinetically at 5 kV for 5 s to extract ~1 nL of homogenate from the homogenate. To enhance microelectrode placement, the capillary inner diameter was enlarged via HF etching. Approximately 2 mm of the polyimide coating was burned off from the capillary to expose the fused silica. The exposed portion of the capillary then was placed in HF for 15 min with a pressure of 3.5 MPa (N₂) through the capillary. Subsequently, the same segment of capillary was placed in a sodium bicarbonate solution to neutralize the acid and then washed with water to remove any excess salt.
Safety Considerations

An in-house-built safety interlock box was utilized to protect the user from high voltage. Since HF could cause severe burns, it was used with extreme care in a fume hood with proper precautions taken.

Results and Discussion

MEKC Separations of Canton S and white Mutant Drosophila Heads

Electrochemical detection methods offer a powerful tool for the determination of biogenic amines within biological samples owing to their high specificity and sensitivity. However when attempting to analyze biogenic amines within the heads of Drosophila melanogaster, an abundance of electroactive species from the eyes, particularly the pigment within the eye (Figure 3-1A), can overwhelm analyses. When homogenized wild-type Canton S fly heads are separated using MEKC, a large separation profile of variable intensities is produced (Figure 3-1B). As neuromodulators are cellularly regulated and are present in low amounts, they produce low (few picoampere) signals. Other signals can be over three orders of magnitude larger, with the peaks at 550 and 1150 s having heights over 10 000 pA, and can end up hindering quantification of less abundant signals with their wide elution times. Under these conditions, the internal standard catechol must be used at a high concentration (100 µM) otherwise; it can become obscured by other peaks in the separation. To help reduce the effects of the high-concentration species and to investigate the effect eye pigment has upon the separation, homogenized white mutant fly heads have been examined (Figure 3-1C) resulting in a vastly different separation. Approximately 90% of the highly expressed and concentrated peaks
observed from the Canton S flies are not observed in the *white* mutant flies producing mostly baseline-resolved peaks of the lower concentration species. This indicates that these abundant electroactive species result from species related to the red eye pigment of the fly. With the removal of these peaks, the internal standard is now easily identified in the separation and can be reduced to a more appropriate level (25 µM).
Figure 3-1 (A) Image of two Drosophila heads that are analyzed in B and C. On the left is a wild-type Canton S, and the right is a white mutant. (B) Electropherogram of three homogenized Canton S Drosophila heads with 25 µM catechol internal standard, peak identified with arrow. Many peaks are observed with some being extremely large. At such a low concentration, the internal standard peak can be difficult to find and quantify. (C) Electropherogram of three homogenized white mutant Drosophila heads with 25 µM catechol as internal-standard; peak identified with arrow. The electropherogram is on the same scale as in B and demonstrates that the intense electroactive peaks observed in B are from the pigment in the eye. (Inset) A magnified view of the electropherogram. The absence of eye-pigment in this sample generates a simpler electropherogram with higher detail within the peaks.
MEKC Separations of Three Types of *Drosophila* Brains

Homogenization of fly brains can be extremely challenging. In contrast to the hard exterior cuticle of the fly head, the fly brain is small and pliant. Additionally, commercially available homogenizers or home-made, miniature, tissue homogenizers are too large and bulky to break apart fly brains making it difficult to obtain a homogeneous sample. Thus, tissue disruption and homogenization is achieved by use of ultrasonication in the work presented here. The 2-mm ultrasonication probe is small enough to reach the solution at the bottom of a 200-µL centrifuge tube. Care is taken to avoid contact with the bottom of the tube with the tip of the sonicator and to carry out sonication in a cold-water bath, to avoid burning a hole in the sample tube. Sonication appears to yield an efficient and systematic method to obtain a uniform sample with no solid cell material visible.

To investigate the biogenic amine differences between *Drosophila* mutants and Canton S, three types of fly brains, wild-type, white mutant, and TH-GFP mutant, have been dissected, homogenized, and separated (Figure 3-2). Figure 3-2 shows the separations of typical *Drosophila* homogenates of three brains for each type of fly. In previous works, homogenates of 50 heads or more were commonly used to analyze the entire fly head. Dissection of that many fly brains is too time consuming creating reproducibility and tissue degradation issues from air oxidation and diffusion. It was possible to reproducibly dissect three brains, which was strategically used here to minimize variation between individual flies.
The separation profiles of all three types of the fly brains shown in Figure 3-2 most closely resemble the separation profile for the white mutant homogenized whole heads (Figure 3-1C inset). The large, overloading peaks, apparently from the eyes, have been eliminated in the wild-type samples, while preserving peaks associated with neuromodulators. A similar effect was also observed with the TH-GFP samples (data not shown). Two immediate differences can be

Figure 3-2 Electropherograms of dissected Drosophila brain homogenates for (A) wild-type, (B) white mutant and, (C) TH-GFP mutant flies. Highlighted are peaks corresponding to dopamine (1), salsolinol (2), octopamine (3), N-acetyltystamine (4), N-acetylseryotonin (5), N-acetyldopamine (6), L-DOPA (7), and the internal standard catechol (8). Some differences in peak intensities are present between these electropherograms, but intensities can be misleading due to changes in electrode efficiency from fouling which is compensated by the internal standard.
seen between all three dissected samples and the *white* mutant heads. The large peak at elution time of 800 s seems to be reduced in the dissected brain samples. More significantly, many of the peaks corresponding to the neuromodulators in the dissected samples have larger peak areas. This is likely a consequence of the faster preparation time and lack of use of a centrifugal filter when preparing the dissected samples. Shorter preparation times help to prevent air oxidation, and analytes might adsorb to the filter excluding them from quantification.

While *white* mutant samples help to show that the eye pigment from Canton S flies produces much of the electroactive species seen in these separations, they result in some biological ramifications. As discussed earlier due to their inability to produce screening pigments in their eyes, *white* mutants experience defective vision when in bright lights. It has also been reported that they suffer from difficulties in movement,\(^7\) deficiencies in learning, and reduced amounts of histamine, dopamine, and serotonin.\(^37\) As such, it is important to quantify and compare these and perhaps other neuromodulators in this mutant species relative to the wild-type species (Canton S).

Quantitative information has been acquired from three independent preparations of the wild-type, *white* mutant, and TH-GFP mutant dissected brains. Neuromodulators dopamine, salsolinol, N-acetyldopamine, L-DOPA, octopamine, N-acetyltlyramine, N-acetylserotonin have been identified by correlating migration times to a 25-\(\mu\)M standard separation run directly after the brain homogenate sample. The average value and SEM for each of these analytes is reported by comparison to the same 25-\(\mu\)M standard separation run directly after the brain homogenate and to a 25-\(\mu\)M internal standard of catechol (Table 3-1).

The small (femtomole) amounts of dopamine, salsolinol, N-acetyltlyramine, N-acetylserotonin, and L-DOPA detected in the *white* mutant all appear to be reduced when compared to wild-type and TH-GFP. Statistical analysis with a two-tailed T-test with equal variances revealed that the *white* mutant is significantly lower in dopamine, salsolinol, and N-
acetyltamine (p < 0.05) and L-DOPA (p < 0.10) relative to Canton S flies. When compared to the TH-GFP flies, the white mutant was significantly lower in salsolinol, N-acetyltamine, and N-acetylserotonin (p < 0.05) and L-DOPA (p < 0.10). It is also interesting to note the differences present in the three fly types between dopamine, L-DOPA, salsolinol, and N-acetyldopamine. The level of dopamine in the white mutant was found to be significantly lower than in the Canton S and TH-GFP flies. As L-DOPA is a precursor and salsolinol is a metabolite to dopamine, it is not surprising that they too are lower in the white mutant than in the other two flies. Also in contrast to previous reports, octopamine is found to not be different between wild-type and white mutant flies, although dopamine is lower in the white mutant fly as previously reported. This could be due to differences in the separation methodology used or possibly the use of dissected fly brains here versus homogenized whole heads.

Table 3-3 Quantification of Biogenic Amines, Precursor, and Metabolites in Canton S, white Mutant, and TH-GFP Drosophila Brains

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Canton S</th>
<th>white mutant</th>
<th>TH-GFP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (fmol)</td>
<td>SEM</td>
<td>Mean (fmol)</td>
</tr>
<tr>
<td>Dopamine</td>
<td>91**</td>
<td>4.8</td>
<td>63</td>
</tr>
<tr>
<td>Salsolinol</td>
<td>140**</td>
<td>14</td>
<td>94</td>
</tr>
<tr>
<td>Octopamine</td>
<td>780</td>
<td>77</td>
<td>700</td>
</tr>
<tr>
<td>N-Acetyltyramine</td>
<td>1600**</td>
<td>300</td>
<td>400</td>
</tr>
<tr>
<td>N-Acetylserotonin</td>
<td>500</td>
<td>190</td>
<td>120</td>
</tr>
<tr>
<td>N-Acetyldopamine</td>
<td>800</td>
<td>190</td>
<td>570</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>1600*</td>
<td>230</td>
<td>900</td>
</tr>
</tbody>
</table>
a Separation of three separate fly culture homogenizations showing quantification of analytes in Canton S, white mutant, and TH-GFP Drosophila brains. Each value corresponds to an amount contained in one brain.

* indicates significantly different from white mutant brain samples with p value less than 0.10

** indicates significantly different from white mutant brain samples with p value less than 0.05

Variability of Biogenic Amines Among Single Drosophila Brains

As previously reported, small sample volumes can be used with capillary electrophoresis making it possible to analyze and study individual fly heads. Analysis of the dissected brain adds a new facet to such analyses, as it provides sample clean up prior to separation by removing many unidentified species that interfere with the separation and detection of biogenic amines and their metabolites. To determine the sensitivity of the MEKC-EC system for single dissected drosophila brains, the biogenic amine and metabolite levels have been calculated for five individual wild-type dissected brains. Individual brains were dissected and ultrasonicated in 1.0 µL of solution containing internal standard, instead of the previously reported 250 nL. The sample volume was increased to the minimum volume that could consistently be used to homogenize fly brains using the 2-mm ultrasonication probe.

Figure 3-3 shows the electropherograms and calculated numeric values for the average amount of analyte detected for each of the five single-fly brain homogenates. The overall separation profile of a single fly-brain homogenate remains consistent between samples with well-defined, baseline-resolved peaks. Migration times correlate
well with little variation between runs, particularly in the beginning of the separations. However, some variation in peak intensities of the more intense peaks is observed, highlighting the potential patterns that may be discerned from analyzing individual animals. Of note are the peaks at 550 s, 700 s, and 1050 s. The peak at 500 s is considerably larger for the first two flies examined vs. the last three, whereas the peak at
700 s is considerably smaller for the first two flies examined vs. the last three. While these two peaks might suggest some co-variation between the five flies, the intensity of the third peak at 1050 s follows a completely different pattern from that of the previous two peaks. Other peaks are also present and show similar features with no covariance between flies. Work to identify these substances is ongoing; however, the pattern is the important feature in the data here, not the absolute chemical identity.

Sample degradation or column changes might be an issue in quantification. To examine this, samples for flies 1-3 were run in duplicate (not shown), and absolute areas for every peak in each of the second runs were found to significantly decrease from the first runs despite storage of samples at -70 °C between runs. This decrease might be attributed to the combination of small sample size (1 μL) and loss due to air oxidation or from transfer during the first injection. Some peaks (~15-20% per sample) were decreased enough that they were no longer discernable from baseline, but relative areas between peaks and the internal standard catechol were found to not significantly vary.

The biogenic amines quantified in Table 3-1 were also measured for these single brain analyses (Figure 3-3B). The average analyte values measured for the five dissected single brains were found not to vary significantly from the Canton-S three-brain homogenates reported in Table 3-1. However, variation still exists among the individual flies for the different analytes and helps to demonstrate the ability of this technique to study individual fly-to-fly variability. For example, the values of L-DOPA, the dopamine precursor, are observed to be present in higher quantities in fly 5 than in fly 2, but the amounts of dopamine and two of its metabolites, N-acetyldopamine and salsolinol, are higher in fly 2 than in fly 5. This is in contrast to L-DOPA levels and might indicate
different levels of metabolism in the two flies. A similar example also occurs between flies 3 and 4, where L-DOPA and dopamine levels are relatively similar between the two flies, but the values of the two metabolites, N-acetyldopamine and salsolinol, are much lower in fly 3 than fly 4. In fact, this trend extends to the two other metabolites, N-acetylttyramine and N-acetylserotonin; for both metabolites, the value measured for fly 3 is lower than in fly 4.

MEKC Separations of *Drosophila* Brain Regions

As this methodology is easily sensitive enough to detect and measure biogenic amines and their metabolites in whole fly brains, individual regions of the brain were examined to push the limits of the technology to levels not achieved before for sampled chemical analysis in this system. In addition, dissection of the *Drosophila* brain into segments offers the ability to obtain quantitative chemical anatomy within the brain. *Drosophila* brains are well categorized physically and physiologically and have many substructures from the overall structure of larger tissues to small clusters of individual neurons, providing the background to match out and map the quantitative chemistry of its regions.

To demonstrate the feasibility of MEKC-EC to detect and assess biogenic amines in these smaller regions, separations of three independent cultures of three brain regions, the two optic lobes, the central brain, and the PPM1 region were examined (Figure 3-4) and quantified (Table 3-2). Figure 3-4A shows a representative optical image of the anterior of a dissected brain. The optic lobes exist on each side of the central brain and
connect the central brain to the compound eyes. The optic lobe processes and integrates visual information from the retina and transmits it to the central brain. Together these two units make up the largest two structures in the fly brain. Conversely, the outer layer of the cortex contains thirteen small clusters of dopamine neuronal cells distributed throughout the *Drosophila* brain. One of these clusters, the PPM1 brain area, contains about five cell bodies, which are not split between the two hemispheres of the brain due to its location along the midline of the brain. To visualize the PPM1 region of the brain, fluorescence microscopy of TH-GFP flies was utilized. Figure 3-4B shows a representative false-color image of the posterior of a dissected brain with GFP-labeled dopamine neurons. The white box outlines the exposed brain region where PPM1 neurons were dissected, while the fluorescent cells outside of the box represent other dopamine neuronal clusters. PPM1 regions were dissected to a depth of about one-quarter of the total depth of the brain (~12 µm) as attempts to dissect smaller lengths were found to be too variable.
Dopamine was examined first. The amount of this transmitter detected in the central brain is close to that reported for the dissected brain homogenates and single dissected brains, whereas very little was observed in the optic lobes. Although there are dopamine neuronal cell bodies present in the optic lobes, they are very few, occur close to the boundary between the optic lobe and central brain, and project into the central brain. As transmitter is typically expected to be higher in the nerve endings, the amount of dopamine present in the optic lobes is likely below the limit of detection for the

**Figure 3-4** (A) Anterior image of an intact, dissected *Drosophila* brain. Brackets show the two optic lobes at the sides and the central brain in the middle. (B) False-color fluorescent image of the posterior of a TH-GFP fly. Green areas indicate cells containing tyrosine hydroxylase, the enzyme responsible for catalyzing the conversion of L-tyrosine to L-DOPA and the rate limiting step in the creation of dopamine. White areas are autofluorescence from larger structures within the brain. The white box denotes the location of PPM1 region, which lies ventrally along midline of the central brain that separates it into its two halves. The 500-µm scale bar is applicable for both panels.
system. The amount of octopamine measured is almost equal in both the central brain and optic lobes. The optic lobes contain 6 of the 100 octopamine neurons in the fly; however, elaborate octopamine arborizations project into the optic lobes, leading to the localization of many nerve endings and likely account for the increase in measured octopamine. Octopamine is not detected in the PPM1 region of the fly brain. If any is present in this region of the brain, it must be at trace levels, below the limit of detection. The total measured amount of dopamine in the central brain was almost five times greater than that found in the PPM1 region, yet when the concentration of dopamine was compared for these two regions, the PPM1 region is twenty-five times as concentrated as the central brain (this is because the PPM1 region is considerably smaller than the central brain).
### Table 3-4  Quantification of Biogenic Amines in the Central Brain, Optic Lobes, and PPM1 Region in Single TH-GFP *Drosophila* Brains

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Location</th>
<th>Measured Amount (fmol)</th>
<th>Calculated Volume (nL)</th>
<th>Concentration (µM)</th>
<th>Mean</th>
<th>SEM</th>
<th>Mean</th>
<th>SEM</th>
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<tbody>
<tr>
<td></td>
<td>Dissected</td>
<td></td>
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<td></td>
<td></td>
<td></td>
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<td></td>
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<tr>
<td></td>
<td></td>
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<td>SEM</td>
<td>Mean</td>
<td>SEM</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dopamine</td>
<td>Central Brain</td>
<td>99</td>
<td>8.7</td>
<td>6.2</td>
<td>16*</td>
<td>1.4</td>
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<td></td>
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<tr>
<td>Dopamine</td>
<td>Optic Lobes</td>
<td>0.3</td>
<td>0.20</td>
<td>4.8</td>
<td>0.6*</td>
<td>0.41</td>
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<tr>
<td>Dopamine</td>
<td>PPM1</td>
<td>21</td>
<td>5.0</td>
<td>0.051</td>
<td>400**</td>
<td>98</td>
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<td></td>
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<tr>
<td>Octopamine</td>
<td>Central Brain</td>
<td>370</td>
<td>73</td>
<td>6.2</td>
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<tr>
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<td>Optic Lobes</td>
<td>310</td>
<td>65</td>
<td>4.8</td>
<td>60</td>
<td>13</td>
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<tr>
<td>Octopamine</td>
<td>PPM1</td>
<td>-</td>
<td>-</td>
<td>0.051</td>
<td>-</td>
<td>-</td>
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<td></td>
</tr>
</tbody>
</table>

*a* Averages of three single, TH-GFP, fly-brain region samples ± standard error of the mean (SEM). Mass amounts are reported in femtomoles per fly head. Brain region volumes were calculated by measuring areas in Figure 3-4 and using 12 µm deep for the PPM1 region and 50 µm deep for the central brain and optic lobe regions.

- values indicate they are below the limit of detection for this technique.

* indicates significantly different from the other marked values with p value of 0.985

** indicates significantly different from the other marked values with p value of 0.999

### Conclusions

A method to analyze dissected whole brains and brain regions for the analysis of biogenic amines and metabolites present in *Drosophila melanogaster* is presented. These results are also compared to earlier work using homogenized whole heads (including the eyes and cuticle). Electroactive species from whole heads produce high intensive signals
in separations that can overlap neurochemical species of interest. Analysis of these isolated dissected regions serves as a form of sample clean up that helps to alleviate this and create a more reproducible and sensitive separation. Utilizing microdissection, the biogenic amines in homogenates of three types of flies, wild-type, white mutant, and TH-GFP, were compared. The white mutant flies were confirmed to have reduced amounts of dopamine, salsolinol, N-acetylttyramine, N-acetylserotonin, and L-DOPA in the isolated brains. This method has also been used to obtain information about spatial differences in biogenic amine levels within different brain regions. These data clearly demonstrate that MEKC-EC has the capability to investigated small brain regions of Drosophila brains.

References

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Chapter 4

Micellar Electrokinetic Capillary Chromatography – Electrochemical Detection of Neuromodulators in Freeze-Dried Drosophila Brains

Introduction

Previous separation methods to study biogenic amines, such as catecholamines (dopamine, norepinephrine, octopamine, etc) and the indoleamine serotonin, have utilized a wide variety of sample homogenization techniques to analyze the fly. Some procedures opt to homogenize a large amount of whole fly heads with glass tissue homogenizers and filter any high mass species with molecular filters.\(^1\)\(^-\)\(^4\) While the increased sample size helps to reduce any individual fly-to-fly variability, a large amount of pigment is left within the sample, which can overwhelm the analysis, hindering the signals from the biogenic amines. Other methods use mutations, such as the *white* mutant\(^5\) which has a down-expressed ABC transporter, which transports the amino and nucleic acid precursors needed to make the pigments in the eyes, to genetically remove the pigment signal,\(^6\) although care must be taken to assure that the mutant has similar activity as its wild-type counterpart, as seen in Chapter 2. Dissection is another method that can be used to effectively and completely remove the signal from the eye-pigment,\(^6\)\(^,\)\(^7\) but dissections of fly heads can be slow and challenging. Due to their small size (< 1 mm\(^3\)), dissections of fly heads require precise tactile dexterity, yet even with a successful dissection, samples can be difficult to accumulate for population analysis, as the brain begins to degrade as soon as it is removed.
Freeze-drying is a process where a sample is frozen and dehydrated under high pressures, forcing the water within the sample to sublimate directly to the gas phase. It has long been used as a method within biological research to preserve both the structure and molecular content of samples. By quickly freezing the sample, the formation of large ice crystals, which can puncture cells, is prevented. When the water is removed, the sample is left behind undisturbed and preserved which can be collected and stored for later analysis. It is this preservation quality of freeze-drying that complements dissecting *Drosophila*. Where the fly’s natural small size and short life-cycle permits a large number of samples to be easily and rapidly available, while preserved brains can be easily and quickly dissected after freeze-drying. As the process helps to significantly degrade enzyme activity (though not completely stop), other molecules found within the brain, such as proteins, phospholipids, and catecholamines, have been quantitatively recovered and analyzed.

In this chapter, methodology to freeze-dry Drosophila for the analysis of biogenic amines using MEKC-EC is discussed. It was found that both the amount of time used to extract the water from the samples and the drying time are vital to optimize to prepare an accurate sample. Using the borate/SDS buffer, freeze-dried fly brain samples could be collected and concentrated to improve the signal of the separation and measure biogenic amine levels within populations of dissected fly brains.
Experimental Section

Reagents

Sodium hydroxide was obtained from Fluka (Buchs, Switzerland) and perchloric acid was purchased from Riedel-de Haën (Morristown, NJ, USA). N-acetylamine dopamine, N-acetylamine octopamine, and N-acetylamine tyramine were obtained from the The National Institutes of Mental Health (NIMH) chemical synthesis and drug supply program (Research Triangle Park, NC, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Separations were performed in 25 mM borate buffer containing 50 mM SDS and 2% 1-propanol with NaOH to produce a calculated pH of 9.52. All standards were prepared as 10 mM stock solutions in 0.1 M perchloric acid and were diluted to the desired concentration with additional 0.1 M perchloric acid.

Drosophila Care and Preparation

Canton-S wild-type Drosophila melanogaster strains were obtained from the Bloomington Drosophila Stock Center (Indiana University, Bloomington, IN, USA) and were maintained in the laboratory. Male flies were cultured on standard potato meal/agar medium and collected for homogenization 4 and 5 days after emerging from pupal cages.

Flies were prepared for CE experiments with methods adapted from protocols previously described. Flies were collected in 15-mL centrifuge tubes, plunged into liquid nitrogen, and vortexed for 60 s in order to separate the heads from the body. Heads were counted, collected, and placed in a 10 mL round bottom flask filled with 10
mL of acetone. The flasks were then frozen in liquid nitrogen and thawed in a water bath three times before being stored at in the freezer (-80 °C) for 2-7 days. Upon removal of the freezer, the round-bottom flasks containing the fly heads and acetone were promptly evaporated with a rotovap under pressure at the lowest speed setting and with a cold water bath.

The fly heads were then placed under an Olympus SZ40 stereoscope. Using small, dissecting forceps (Fine Science Tools, Heidelberg, Germany), the cuticle was cracked and brain extracted. Dissected brains were collected in 200-µL PCR tubes (VWR International, West Chester, PA, USA) with 1.0 µL of 0.1 M perchloric acid/25-100 µM catechol (internal standard) and centrifuged (VWR International, Galaxy Mini, West Chester, PA, USA) at 6000 RPM for 30 s. The heads were then ultrasonicated (Sonics & Materials, Inc, Satigny, Switzerland) with a 2 mm microtip at 100% amplitude for 120 s in a cold-water bath. The sample was centrifuged for 30 s for collection and promptly injected onto the capillary.

**Instrumentation Procedures and Analysis**

The CE system with end-column amperometric detection utilized in this study was built in-house and has been described previously. Briefly, 45-50 cm of fused-silica capillary with an outer diameter of 151 µm and inner diameter of 14 µm (Polymicro Technologies, Phoenix, AZ) was used for the separations. Capillaries were filled with separation buffer, previously described, using a stainless steel reservoir with applied N₂ pressure (3.5 MPa). Injections were then performed electrokinetically at 5 kV for 5 s to
extract ~1 nL of sample from the homogenate. To enhance microelectrode placement, the capillary inner diameter was enlarged via HF etching as previously described. Briefly, approximately 2 mm of the polyimide coating was burned off from the capillary to expose the fused silica. The exposed portion of the capillary then was placed in HF for 15 min with a pressure of 3.5 MPa (N₂) through the capillary. Subsequently, the same segment of capillary was placed in a sodium bicarbonate solution to neutralize the acid and then washed with water to remove any excess salt.

Amperometric electrochemical detection was carried out with a two-electrode format in which a 5-µm carbon fiber microelectrode which was held +0.75 V versus a Ag/AgCl reference electrode (Harvard Apparatus, Holliston, MA, USA). The current was measured using a Keithley model 427 current amplifier (Cleveland, OH, USA) and recorded at 10 Hz using LabView 8.0 (National Instruments, Austin, TX, USA) software, written in-house. Microsoft Excel (Redmond, WA, USA) was used to generate the electropherograms and statistics from the data. Data analysis was performed using Peakfit Separation and Analysis Software v4.11 (SPSS Inc., Chicago, IL, USA).

**Safety Considerations**

An in-house-built safety interlock box was utilized to protect the user from high voltage. Since HF could cause severe burns, it was used with extreme care in a fume hood with proper precautions taken.
Results and Discussion

Freeze-Drying Drosophila Heads

Freeze-dried, fly heads (Figure 4-1) were removed from the freezer and examined under a stereoscope to examine morphology. Visually, the fly heads appeared well preserved with no apparent fractures within the cuticle or pieces missing from the head. When microforceps were used to disrupt and remove the cuticle, it was found to easily crack and separate, having become significantly more brittle than a non-freeze-dried specimen. Once the cuticle had been opened, the brain and optic nerves were then lifted out and collected for analysis for MEKC.

Freeze-drying makes it easier to dissect Drosophila heads faster. In non-freeze-dried samples, the cuticle is quite springy and resistant to being punctured; therefore, significant time is spent positioning the forceps to grab and hold the head properly so that tactical places along the proboscis can be cut to open the cuticle. The two halves of the cuticle must be pulled apart from one another with the force applied being enough to completely expose the brain but gently enough to ensure that the brain is left intact. In fresh specimens care must also be taken not to disrupt the compound eyes whose pigment upon agitation can dissolve into the dissecting solution and cover the brain. Even after a full dissection, many air sacs can still surround the brain, which can impede visual confirmation that the brain is indeed whole. Conversely, in a freeze-dried fly head, a single pair of forceps can gently be used to poke holes in the cuticle and remove enough of this structure to reach the brain. The surrounding air sacs attached to the brain tissue and inner-wall of the cuticle are found to be disconnected after freeze-drying allowing the
intact brain to easily be lifted from the shell of the cuticle. Even if pieces of the compound eye are found attached to the optic nerve (Figure 4-l c and d), these pieces are generally large flakes that can easily be lifted off of the brain sample with forceps.

**Optimization of the Freeze-Drying Process**

To optimize the freeze-drying process, fly heads with various drying and freezer-storage times were investigated by examining catecholamine analysis of these samples with MEKC-EC (Figure 4-2). Previous work carried out using freeze-drying methodologies for *Drosophila* involved storing the frozen (in acetone) fly heads for 7-10 days before removing them from the freezer to dry off the acetone. They were then placed on filter paper to let the acetone to evaporate, which can take up to an hour, or dried under a vacuum (0.1 torr) for 8 to 72 hours. As some enzymatic activity is still present after the freeze-drying process, however low, a faster drying approach seemed to be more appropriate. Samples of five frozen *Drosophila* heads were stored for 2 days at -80 °C and dried for 120, 60, 30, 20, 10, and 5 min, with the 10 mL of acetone being completely evaporated after approximately 10 min. Samples dried for 5 min were found to be too wet with acetone, and as such the cuticle was too pliable to see any improvement in dissection time. Conversely, samples dried for 120, 60, and 30 min were found to have cuticles easy to open, but only a small of amount of “white power” was found on the inside of the cuticle. These brains had completely lost their shape and could not be collected for analysis. Possibly such an extended application of high pressure
began to sublime other molecules within the head causing the brain to crumble and lose their structure.

Brain samples for the 10- and 20-min drying times could be retrieved and analyzed with MEKC-EC (Figure 4-2a,b). The separations following the evaporation time of 10 min have more peaks and larger amounts in peaks with similar elutions time when compared to the separation that had been dried for a longer time. Peaks missing in the sample with the longer evaporation time include those at 450, 475, 490, 525, 600, and 800 s, and the peaks that are present have significantly decreased peak areas (>50%). A balance between enough drying time to remove the acetone but not so much as to degrade

Figure 4-1 Image of a) a freeze-dried fly central brain and b) a freeze-dried fly head. Visually the freeze dried head does not appear to be different from a non-dried counterpart. c) A dissected cuticle with an undamaged brain specimen containing both optic lobes and central brain. d) A dissected cuticle and accompanying brain that is missing one of its optic lobes.
the sample must be achieved, and the ten min of drying time was chosen as optimal for these experiments.

Previous work with freeze-drying of fly brains\textsuperscript{9, 10} also involved longer storage time (with freezer times of 7-10 days before drying). This long storage time was used to allow the acetone to slowly extract water and other hydrophilic molecules from the cells. When five-brain samples were stored for 7 days using the method presented here and dissected, the extracted brains were orange in appearance suggesting that the pigment was being extracted from the eyes into the brain area. When these samples were analyzed using MEKC-EC (Figure 4-2c), the separations were found to have more peaks and poorer resolution of those peaks, looking more like samples from full-head preparations as shown in Chapter 2. In samples sitting in frozen acetone for an extended time, the elution of molecules seemed to take place earlier, as observed with the doublet found at 925s in Figure 4-2a eluting at 850s in Figure 4-2c. Possibly, these newly extracted compounds could be interacting with the rest of the sample, causing less interaction with the SDS micelle pseudo stationary phase and resulting in these faster separation times. Samples stored for two days or less were found to have no discoloration and were used for all subsequent analyses.
Figure 4-2 Electropherograms of five freeze-dried dissected fly brains that have been (a) stored for 2 days in the freezer and dried for 10 min, (b) stored for 2 days in the freezer and dried for 20 min, and (c) stored for 7 days in the freezer and dried for 10 min. A unique electrode was used for each separation, but the area for the internal standard, catechol, for each separation was found to range only 5% between runs.
Increasing Brain Numbers for Population Analysis

Drosophila’s small size (~1 mm) and short life cycle (15 days) allows for multiple flies to be homogenized into larger sample sizes which not only helps to even out the variability seen between individual flies but helps to concentrate samples. Dissection of non-freeze dried flies is not a trivial matter. Not only is the cuticle difficult to break apart, but exposure to the dissecting solution for periods greater than 15 min can lead to brain swelling and increased tissue tearing from osmotic pressure on the buffer into the cells. Moreover, hand-dissected brains are not preserved and can have intact enzymatic activities. As such, separations of samples containing more than three dissected brains have been too difficult to carry out, even by someone highly skilled in dissections.

Here, freeze-drying is shown to alleviate these obstacles by preserving and making the dissections faster. A key aspect of freeze-drying is that it allows use of much smaller volumes to dissolve the brains and their metabolites. Samples containing 1, 3, 5, 10, and 15 brains in 1 µL of 50 µM catechol/0.1 M HClO₄ were analyzed with MEKC-EC to evaluate the ability of the system to resolve samples containing varying numbers of fly brains (Figure 4-3). As the brain number increased, analyte peaks become more resolved and intense permitting better identification and quantification of these peaks. This effect is clearly apparent for the doublet that occurred after the internal standard catechol (~900s). These peaks steadily increased in size with increased brain number. Quantitative data is presented below for several separations. For the 1, 3, and 5 brain samples, these peaks were not consistently resolved from one another, and when they did resolve, these peaks had an R < 1.5. As the sample concentration was increased to 10 and
15 brains, these two analytes became resolved from one another with an \( R > 1.5 \). Possibly the increased number of brains in these 1 µL samples, and therefore increased concentration of
these analytes, provided increased interaction with the SDS micelle pseudo stationary phase to compensate for any variability in the sonication process and is reflected here to create the increase resolution.

Measuring Neuromodulators in Freeze-Dried Wild-type Drosophila Brain Homogenates

To demonstrate the applicability of using this method in bioanalysis, biogenic amines and metabolites have been separated and quantified in homogenates containing 15 freeze-dried brains of Canton-S Drosophila. Analyte peak identification was performed using spiked samples and comparisons of 50 µM standard separations run before and after the homogenate separation. Figure 4-4 displays sample electropherograms of a 50-µM standard and the corresponding separation homogenate of 15 Drosophila brains in 1 µL that was run directly after the standard sample. Peaks corresponding to octopamine (4), N-acetylserotonin (5), N-acetyltymamine (6), N-acetyldopamine (7), l-DOPA (8), and tyramine (10) were easily identifiable and had intensities far above baseline (50-200 pA), which are far larger than those shown in Chapters 2 and 3. Octopamine was found to co-elute with another, unidentified peak that occurred at 490 s, which prevented it from being repeatedly quantified.
If a smaller number of brains were used, such as 5 or 10, these analytes would still have been identifiable. The increased concentration of metabolites (and resulting increased separation resolution) clearly facilitates the identification process and helps to make quantification more accurate. It has also been hypothesized that the acetone used to freeze the fly heads can help to extract hydrophilic species from the cells, and extracted analytes such as these would then appear in greater quantities. These analytes help to demonstrate that MEKC-EC can be used to analyze biogenic amines and metabolites in freeze-dried biological samples.

Figure 4-4 Electropherogram of a (bottom) freeze-dried dissected homogenate of 15 brains, and (top) the accompanying 50 µM standard separation used to identify the following peak identities: (1) dopamine, (2) salsolinol, (3) N-acetyloctopamine, (4) octopamine, (5) N-acetylserotonin, (6) N-acetyltityramine, (7) N-acetyldopamine, (8) l-DOPA, (9) catechol, and (10) tyramine.
These samples were also analyzed for dopamine (1), salsolinol (2), N-acetyloctopamine (3), and octopamine (4). Peaks were found to be difficult to identify as peak areas did not increase as much as the other analytes as the samples were concentrated samples. Peaks corresponding to dopamine and N-acetyloctopamine were identifiable but had intensities close to baseline (<10 pA), while salsolinol was not clearly discernable from the baseline. It is not entirely clear why these analytes are less prevalent under these conditions. One possibility is that the acetone, which could be helping to extract several analytes, might lead to loss of the more volative analytes as they might evaporate with the acetone. This is further supported by the separation times as all three of these species elute within 50 s of one another and are the first species eluted. Hence, these analytes interact with the SDS micelle pseudo stationary phase similarly and have similar physical properties, hydrophobicities. Thus, they might be expected to have similar volatilities.

Quantitative information has been acquired from four independent preparations of 15 homogenized freeze-dried Canton-S brains. Dopamine, salsolinol, N-acetyloctopamine, N-acetylserotonin, N-acetyltyramine, N-acetyldopamine, L-DOPA, tyramine have been quantified. The average value and SEM for each of these analytes per brain is reported by comparison to the same 50-μM standard separation run directly before the brain homogenate and to a 50-μM internal standard of catechol (Table 4-1).
Table 4-1  Quantification of dopamine, salsolinol, N-acetyloctopamine, N-acetylserotonin, N-acetyltyramine, N-acetylDOPA, and tyramine in homogenates of 15 freeze-dried brains. Values reported for each analyte include the femtomole amount of the analyte (C) and mean ± the SEM for the four fly samples.

<table>
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<th>Dopamine</th>
<th>N-acetyl octopamine</th>
<th>N-acetyl serotonin</th>
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<tr>
<td>1</td>
<td>22</td>
<td>10</td>
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<tr>
<td>4</td>
<td>11</td>
<td>12</td>
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</tr>
<tr>
<td>Mean ± S.E.M.</td>
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<td>9 ± 1.0</td>
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<table>
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<td>1</td>
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<tr>
<td>3</td>
<td>687</td>
<td>1090</td>
<td>1550</td>
<td>934</td>
</tr>
<tr>
<td>4</td>
<td>594</td>
<td>1190</td>
<td>1670</td>
<td>951</td>
</tr>
<tr>
<td>Mean ± S.E.M.</td>
<td>640 ± 20</td>
<td>1110 ± 36</td>
<td>1660 ± 38</td>
<td>950 ± 14</td>
</tr>
</tbody>
</table>

Conclusions

An improved method for the analysis of freeze-dried brain homogenates for the analysis of biogenic amines and metabolites present in Drosophila melanogaster has been developed. Previous methodologies for freeze-drying of fly heads proved unsatisfactory, so extraction and drying times were quantitatively explored and optimized. Drying for too long produced low signals apparently owing to sample loss, while long freezer storage times led to samples over-extracted by acetone solvent. After too much time, unidentified and undesirable electroactive compounds from the eyes of the fly also began migrating into brain, defeating one purpose of using dissected samples – eliminating peripheral interferences. Utilizing the faster dissection time that freeze-
drying affords, the number of brains in a homogenate was increased to concentrate the sample. This helped to not only increase the amount of signal within the electropherograms but also the resolution between analytes. Samples containing fifteen preserved brains were analyzed for their neuromodulator content. N-acetylserotonin, N-acetyltlyramine, N-acetyldopamine, L-DOPA, and tyramine were found to all correspond well with previous dissected studies.

References

Chapter 5

Chapter 6 Optimization of Capillary Electrophoresis-Electrospray Ionization-Time of Flight Mass Spectrometry for the Examination of Drosophila Analytes

Introduction

The driving force for coupling CE to MS is the detailed structural information that is provided by MS. Capillary electrophoresis has been coupled on-line to MS with electrospray ionization (ESI),\(^1\) and atmospheric pressure chemical ionization,\(^2\) and off-line with matrix assisted laser desorption ionization.\(^3\) ESI is the most widely used of these ionization techniques due to its simplicity, high ionization efficiency, and the ability to produce multiply-charged ions.\(^4\) A coaxial liquid sheath is one method for interfacing CE and ESI-MS where three coaxial capillaries are placed at the interface of CE and MS.\(^1\) The innermost capillary is the fused-silica separation capillary, while the middle capillary delivers the sheath liquid and is made of stainless steel. The stainless-steel outer capillary delivers a nebulizing gas, which aids in droplet formation.\(^5\) Sheath liquids usually consist of water, organic solvent (e.g. methanol), and an electrolyte. They serve to provide electrical contact between the CE and MS and increase the liquid flow to the ESI to ensure stable ion production.\(^1\) However, sheath liquids can dilute analytes, which reduces sensitivity,\(^6,\)\(^7\) and cannot completely compensate for nonvolatile separation buffers.

Previous conditions for the analysis of biogenic amines in *Drosophila* have exploited the selectivity provided by MEKC with EC detection. Separation conditions included either a 10
mM TES/30 mM SDS/2% 1-propanol separation buffer at neutral pH\textsuperscript{8,9} or a 10 mM TES/30 mM SDS/2% 1-propanol separation buffer at neutral pH or a 25 mM borax/50 mM SDS/2% 1-propanol separation buffer at basic pH.\textsuperscript{10, 11} However, to analyze these samples with CE with MS, both buffers would severely limit analyte detection as the continuous introduction of nonvolatile buffers and surfactants into ESI-MS can result in a significant loss of electrospray efficiency (ion suppression) and even ion source contamination. As such, a more volatile buffer was chosen.

In this chapter, more volatile separation buffers are investigated for their ability to separate biogenic amines with the goal of quantification within Drosophila. The pH of these buffers were then optimized to ensure unique masses of biogenic amines were available and to offset the loss of selectivity seen with MS. Ten previously studied biogenic amines/metabolites, three new neurotransmitters, and a drug, were all then analyzed with CE-ESI-TOF.

**Experimental Section**

**Reagents**

N-acetylamine dopamine, N-acetylamine octopamine, and N-acetylamine tyramine were obtained from the the National Institutes of Mental Health (NIMH) chemical synthesis and drug supply program (Research Triangle Park, NC, USA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Separations were performed in 10 mM ammonium formate or 10 mM ammonium acetate buffer with various concentrations of ammonium hydroxide. All standards were prepared as 10 mM stock solutions in water, diluted to the desired concentration, and frozen to prevent air oxidation.
Buffer Optimization

The CE system with end-column amperometric detection that was used for the buffer optimization in this study was built in-house and has been described previously.\textsuperscript{9,12} Briefly, 45-50 cm of fused-silica capillary with an outer diameter of 151 µm and inner diameter of 14 µm (Polymicro Technologies, Phoenix, AZ) was used for the separations. Amperometric EC detection was carried out with a two-electrode format in which a 5-µm carbon fiber microelectrode was held $+0.75$ V versus a Ag/AgCl reference electrode (Harvard Apparatus, Holliston, MA, USA). The current was measured using a Keithley model 427 current amplifier (Cleveland, OH, USA) and recorded at 10 Hz using a LabView 8.0 (National Instruments, Austin, TX, USA) interface, written in-house. Microsoft Excel (Redmond, WA, USA) was used to generate the electropherograms and statistics. Data analysis was performed using Peakfit Separation and Analysis Software v4.11 (SPSS Inc., Chicago, IL, USA).

Buffer solutions were filtered with a 0.2 µm nylon filter (Alltech, Deerfield, IL) prior to use. Capillaries were filled with separation buffer using a stainless steel reservoir with applied N\textsubscript{2} pressure (3.5 MPa) and washed between changes in buffer with deionized water (18.0 MΩ) for ~15 min. Injections were then performed electrokinetically at 5 kV for 5 s. To enhance microelectrode placement, the capillary inner diameter was enlarged via HF etching as previously described.\textsuperscript{12} Approximately 2 mm of the polyimide coating was burned off from the capillary to expose the fused silica. The exposed portion of the capillary then was placed in HF for 15 min with a pressure of 3.5 MPa (N\textsubscript{2}) through the capillary. Subsequently, the same segment of capillary was placed in a sodium bicarbonate solution to neutralize the acid and then washed with water to remove any excess salt. Typically, the same capillary was employed for
any single set of experiments such as all the samples in the set of standard additions or calibration curve.

The buffers with different pH values used to optimize separations were made as follows. Five liters of 10 mM ammonium formate were made and divided into equal 0.250 L portions. Small amounts of ammonium hydroxide were added to each portion sequentially increasing the concentration and monitored with a pH meter International, LLC, West Chester, PA, USA) from pH 7.5 to 9.5 (by increments of 0.1 pH units).

CE-MS

Analysis of neuromodulators using CE-MS was performed using a modified Waters Capillary Ion Analyzer Model Quanta 4000E (Milford, Massachusetts) coupled to a Micromass Quattro triple quadrupole (Cary, NC) with electrospray ionization (ESI). To modify the CE instrument, a small hole was drilled into the side of the instrument, and a 1 cm piece of rubber tubing was placed inside the hole. One end of a 60-cm long, separation capillary with an outer diameter of 150 µm and inner diameter of 50 µm (Polymicro Technologies, Phoenix, AZ) was placed in the injection end of the CE. The other end was then threaded through the rubber tubing in the side of the CE and connected to the CE sprayer of the mass spectrometer. There it met a 10-cm transfer capillary with an outer diameter of 200 µm and inner diameter of 50 µm that was used for nebulization. A grounding wire was then connected from the CE sprayer to the ground of the CE instrument so that the CE instrument would operate normally. The potential over the transfer capillary was 4.0 kV, whereas the cone voltage was set for 40 V for reduced fragmentation. The source block temperature was held at 80 °C, and the nebulization gas was
held between 0.5 and 1 bar. Analyte detection was performed using a Micromass QToF, quadrupole time-of-flight mass spectrometer with ESI scanning between m/z 35 and 300.

The optimized ammonium formate buffer solutions were filtered with a 0.2 µm nylon filter (Alltech, Deerfield, IL) prior to use. Capillaries were filled with separation buffer using a Hamilton syringe. Injections were then performed electrokinetically at 5 kV for 5 s. To compensate for the decreased amount of sample from the CE, a coaxial sheath flow of 49.5% methanol, 49.5% water, 1% formic acid was implemented. A syringe pump (KD Scientific, Holliston, MA) was used to control volumetric flow of sheath flow solution via 5 mL plastic syringes at a rate of 0.8 µL/min.

Results and Discussion

Optimization of Separation Conditions for Biogenic Amines in Drosophila

Before carrying out analysis with the CE-ESI-MS, it was necessary to optimize the separation conditions of a more volatile buffer for which CE-EC was used. Previous work demonstrated that it is possible to resolve 23 biogenic amines with a 25 mM borax/50 mM SDS/2% 1-propanol separation buffer at a calculated pH = 9.66, 11 and while such a high degree of resolution is not as crucial when using MS, some degree of separation is still useful when analyzing these compounds. Some biogenic amines are isomers of one another, e.g. dopamine and octopamine, which only differ by the placement of a single hydroxyl group. If these analytes coelute, it would be impossible to qualify, let alone quantify, these species given their identical masses and using their molecular ion. Additionally, despite use of the dissection techniques discussed in Chapters 3 and 4, unidentified analytes are still present in the Drosophila samples,
and MS is a more general technique, observing a larger number of compounds, than EC. Unidentified peaks could potentially coelute with the biogenic amines, and thus the optimization of the buffer helps to mitigate this.

Test mixtures containing dopamine, l-DOPA, N-acetyldopamine, salsolinol, octopamine, N-acetyloctopamine, tyramine, N-acetyltamine, serotonin, and N-acetylserotonin were separated with running buffers of 10 mM ammonium with various counter ions: chloride, formate, and acetate. The ammonium ion was chosen as it can be deprotonated to become highly volatile ammonia, making it suitable for mass spectrometry, while the different anions were compared to find the one with the best resolution capability. Separations using the 10 mM ammonium chloride separation buffer proved to be too unstable to provide a reliable separation. Baselines were erratic and it was not possible to identify peaks corresponding to the biogenic amines. Using the ammonium formate or acetate as counterions yielded separations with stable baselines having high signal to noise ratio and sharp peaks (Figure 5-1).

Both of these separations had profiles containing three sets of peaks: one set at ~200 s, one close to the system peak, and one set eluting at times greater than 300 s. All eleven analytes were found to elute from the column before 400 s, which is earlier than when the first analyte, dopamine, would elute from the column with the borate/SDS separation buffer. This and the decreased resolution highlight the effects of removal of the micellar pseudostationary phase. As the analytes are no longer able to interact with the micelles, they pass through the column considerably faster. The different analytes appeared to separate based on how substituted their amine was. Thus, all of the analytes containing a primary amine (dopamine, l-DOPA, octopamine, tyramine, and serotonin) eluted in the first set of peaks around 200 s and before the background-electrolyte induced system peak, indicating a positive charge. Peak 2 has been
identified as N-acetyldopamine, whereas the other secondary amines have been identified to elute within peak 3 by injecting individual standards. Although it seems surprising that dopamine did not coelute with the other N-acetylated biogenic amines in peak 3, a similar pattern between dopamine and the other primary amines later appeared when the separations were further optimized (vide infra).

Figure 6-1 CE-EC electropherograms of 50 µM for ten biogenic amines using a 14-µm-i.d. capillary to compare running buffers 10 mM ammonium formate and 10 mM ammonium acetate. Peaks correspond to: 1) tyramine, serotonin, octopamine, dopamine, and L-DOPA 2) N-acetyldopamine and 3) N-acetyltyramine, N-acetylserotonin, N-acetyloctopamine, and salsolinol.
While the ammonium formate and acetate running buffers yielded the same number of peaks with the same analytes within each peak, the ammonium formate separations clearly showed better peak resolution. Peaks were also sharper and had a better signal to noise ratio. Four distinct peaks belonging to five analytes were identifiable in the first set of peaks at ~200 s in the ammonium formate separation, compared to the doublet in the ammonium acetate separation. The most dramatic example of peak widths was with N-acetyldopamine (peak 2) where the ammonium formate separation had a half-width of 2 s. The same peak for the ammonium acetate separation had a half-width of 29 s, almost 15 times wider. Considerable tailing was observed for peak 3 in the ammonium acetate separation indicating possible separation of the species there, but this result was found to be variable with repeated runs becoming as narrow as the peak found in the ammonium formate separation.

The ammonium formate separation was further optimized by altering its pH using a similar protocol to that used in Chapter 2. Incremental amounts of hydroxide ion were added to the running buffer from pH 7.5 to 9.5. Ammonium hydroxide was used instead of sodium hydroxide to minimize the amount of nonvolatile compounds present within the buffer. The pH of solutions was monitored with a pH meter since there was no SDS present to interfere with readings. Representative electropherograms of these separations are shown in Figure 2-2.

Resolution of analytes increased with increasing pH until a value of 9.375 where peaks began to broaden significantly. For the first pH value of 7.540, the same set of three peaks was observed with tyramine, serotonin, octopamine, dopamine, and L-DOPA eluting first. This was then followed by a sharp N-acetyldopamine peak and then a single peak containing N-acetyltyramine, N-acetylserotonin, N-acetyloctopamine, and salsolinol. As the pH was increased to 8.503, the N-acetyldopamine peak began to coelute with the first set of peaks. These first six
peaks still had poor resolution, eluting close to one another, but were resolved enough to identify individual peaks for tyramine, serotonin, l-DOPA, and N-acetyldopamine. Further increasing the pH to 9.008 resulted in a dramatic change in resolution for dopamine/l-DOPA, and N-

Figure 6-2 Selected standard separations of 50 μM (1) tyramine, (2) serotonin, (3) octopamine, (4) dopamine (5) L-DOPA, (6) N-acetyldopamine, (7) N-acetyloctopamine, (8) N-acetylserotonin, (9) N-acetyltiramine, and (10) salsolinol with increasing pH to optimize separation resolution between analytes. Amounts of [OH⁻] aliquots were measured with a pH meter from 7.540 to 9.563. All separations were run sequentially on the same 50-cm piece of capillary using the same electrode at 15 kV with a 15-min wash of deionized water between buffer changes.
acetyldopamine providing baseline resolved peaks. Baseline resolution was achieved for octopamine at a pH of 9.375, but at the cost of resolution between the dopamine/l-DOPA and N-acetyldopamine peaks. At this point, peaks began to become wider and be less sensitive. Since serotonin and octopamine have significantly different masses, it was decided that a pH of 9.129 would be optimal for CE-MS experiments. A listing of the separation efficiencies for the different pH values of ammonium formate buffer are given in Table 5-1 for those peaks where these could be calculated.

Table 6-1  Calculated resolutions for peaks identified in the electropherograms at different pHs of ammonium formate running buffer in Figure 5-2. Analytes that are present in the peaks used for the resolution calculations are referred by the numbers used there.

<table>
<thead>
<tr>
<th>pH of Buffer</th>
<th>Analyte(s) Identified in First Compared Peak</th>
<th>Analyte(s) Identified in Second Compared Peak</th>
<th>Resolution</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.54</td>
<td>1,2,3,4,5</td>
<td>6</td>
<td>3.7</td>
</tr>
<tr>
<td>7.54</td>
<td>6</td>
<td>7</td>
<td>32.8</td>
</tr>
<tr>
<td>8.503</td>
<td>1</td>
<td>2</td>
<td>0.7</td>
</tr>
<tr>
<td>8.503</td>
<td>2</td>
<td>3,4</td>
<td>0.6</td>
</tr>
<tr>
<td>8.503</td>
<td>3,4</td>
<td>5</td>
<td>0.8</td>
</tr>
<tr>
<td>8.503</td>
<td>5</td>
<td>6</td>
<td>0.7</td>
</tr>
<tr>
<td>8.503</td>
<td>6</td>
<td>7</td>
<td>15.5</td>
</tr>
<tr>
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<td>1.0</td>
</tr>
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</tr>
<tr>
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<td>2,3</td>
<td>4,5</td>
<td>2.3</td>
</tr>
<tr>
<td>9.129</td>
<td>4,5</td>
<td>6</td>
<td>2.9</td>
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<td>7</td>
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<td>2</td>
<td>3</td>
<td>1.1</td>
</tr>
<tr>
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<td>3,4</td>
<td>5</td>
<td>4.5</td>
</tr>
<tr>
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<td>5</td>
<td>6</td>
<td>1.6</td>
</tr>
<tr>
<td>9.375</td>
<td>6</td>
<td>7</td>
<td>4.8</td>
</tr>
<tr>
<td>9.57</td>
<td>1</td>
<td>2</td>
<td>0.6</td>
</tr>
<tr>
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<td>2</td>
<td>3</td>
<td>1.3</td>
</tr>
<tr>
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<td>4,5</td>
<td>6</td>
<td>0.6</td>
</tr>
<tr>
<td>9.57</td>
<td>7</td>
<td>7</td>
<td>1.0</td>
</tr>
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</table>
CE-ESI-TOF Analysis of Biogenic Amines

Unique m/z ratios for each of the ten previously optimized neuromodulators and four new analytes, three neurotransmitters glycine, glutamate, and γ-aminobutyric acid (GABA) and the dopamine transporter blocker methylphenidate, were determined by individually running each analyte through the running buffer of the CE-ESI-TOF instrument. Glycine, glutamate, GABA, and methylphenidate are not electroactive and were not detectable using CE-EC. By running the analytes through the system with the running buffer, any background signal from the ammonium formate running buffer or methanol/water/formic acid sheath flow liquid that might hinder identification were also included within the spectra obtained (Figure 7-3). Unique m/z ratios are indicated in each spectrum with an asterisk while their corresponding fragments are listed for each analyte in Table 5-2. The only overlaps in these unique m/z ratios are observed for the dopamine/octopamine and possibly l-DOPA/salsolinol pairs. Neither are of concern as neither pair coelutes in the optimized separation.
Figure 6-3 Representative CE-ESI-TOF spectra obtained for the fourteen analytes in running buffer and sheath flow liquid. The (*) highlights the unique mass-to-charge fragments listed in Table 5-2.
Table 6-2 Table of characteristic neurotransmitter and metabolite unique mass-to-charge ratios.

<table>
<thead>
<tr>
<th>Neurotransmitter/Metabolite</th>
<th>Fragment</th>
<th>Mass (m/z)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dopamine</td>
<td>C₈H₁₁NO⁺</td>
<td>137</td>
</tr>
<tr>
<td>Dopamine</td>
<td>C₈H₁₂NO₂⁺</td>
<td>154</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>C₉H₁₁NO₃⁺</td>
<td>181</td>
</tr>
<tr>
<td>L-DOPA</td>
<td>C₉H₁₂NO₄⁺</td>
<td>198</td>
</tr>
<tr>
<td>N-acetyldopamine</td>
<td>C₁₀H₁₄NO₃⁺</td>
<td>197</td>
</tr>
<tr>
<td>Salsolinol</td>
<td>C₁₀H₁₁N⁺</td>
<td>145</td>
</tr>
<tr>
<td>Salsolinol</td>
<td>C₁₀H₁₃NO⁺</td>
<td>163</td>
</tr>
<tr>
<td>Salsolinol</td>
<td>C₁₀H₁₄NO₂⁺</td>
<td>180</td>
</tr>
<tr>
<td>Octopamine</td>
<td>C₈H₁₁NO⁺</td>
<td>137</td>
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<tr>
<td>Octopamine</td>
<td>C₈H₁₂NO₂⁺</td>
<td>154</td>
</tr>
<tr>
<td>N-acetyloctopamine</td>
<td>C₁₀H₁₄NO₃⁺</td>
<td>197</td>
</tr>
<tr>
<td>Tyramine</td>
<td>C₈H₁₁N⁺</td>
<td>121</td>
</tr>
<tr>
<td>Tyramine</td>
<td>C₈H₁₂NO⁺</td>
<td>138</td>
</tr>
<tr>
<td>N-acetyltyramine</td>
<td>C₁₀H₁₂NO₂⁺</td>
<td>181</td>
</tr>
<tr>
<td>Serotonin</td>
<td>C₁₀H₁₂N₂⁺</td>
<td>160</td>
</tr>
<tr>
<td>Serotonin</td>
<td>C₁₀H₁₃N₂O⁺</td>
<td>178</td>
</tr>
<tr>
<td>N-acetylserotonin</td>
<td>C₁₂H₁₆N₂O₂⁺</td>
<td>220</td>
</tr>
<tr>
<td>Glycine</td>
<td>C₂H₄NO₂⁺</td>
<td>76</td>
</tr>
<tr>
<td>Glutamate</td>
<td>C₅H₁₀NO₄⁺</td>
<td>148</td>
</tr>
<tr>
<td>GABA</td>
<td>C₄H₁₀NO₂⁺</td>
<td>104</td>
</tr>
<tr>
<td>Methylphenidate</td>
<td>C₅H₁₀N⁺</td>
<td>84</td>
</tr>
<tr>
<td>Methylphenidate</td>
<td>C₁₄H₂₀NO₂⁺</td>
<td>235</td>
</tr>
</tbody>
</table>
Conclusions

CE-ESI-ToF mass spectrometry offers a unique opportunity for the analysis of neurotransmitters, metabolites, and drugs within the brains of *Drosophila*. Separations for the analysis of several neuro-related analytes of interest with CE-ESI-TOF have been optimized by choice of a volatile running buffer at a specific pH that ensures that a unique m/z ratio is available for each analyte when they are detected. Neurologically, these separations when coupled to investigations of *Drosophila* could help us to further understand the roles these neurotransmitters play in the fly brain. CE-EC is selective in that only electroactive metabolites and transmitters can be detected. This reduces the overall background signal and simplifies the separation, but it can also prevent the analysis of many analytes of interest. Thus for example, the major inhibitory neurotransmitter, GABA, and major excitatory neurotransmitter, glutamate, are not detectable with CE-EC. CE-MS allows for a wider variety of analytes to be studied including the non-electroactive neurotransmitters, neuropeptides, and drugs, without relinquishing the small sample size and fast analysis times, although perhaps sacrificing detection limit some. Unknown peaks can be identified, while known peaks verified. Recently, the Ewing lab has found that oral consumption of methylphenidate is capable of blocking the *Drosophila* dopamine transporter and inhibits the actions of directly applied cocaine (data unpublished). As the pharmacokinetics and half-life of methylphenidate are slower and longer, respectively, than those for cocaine in the human brain (based on the duration of the blocked dopamine transporter), methylphenidate offers a potential medication for cocaine addiction treatment. An important aspect of this line of investigation is to evaluate the effective concentration of methylphenidate in the brains of flies by oral consumption. Analysis of *Drosophila* brains with CE-ESI-MS could provide a potential method to measure this
concentration and eventually help to translate what concentrations would be applicable in humans.

References

Chapter 6

Future Directions for Quantifying Neurochemicals in *Drosophila* Using Separations

Summary and Conclusions

Given *Drosophila*’s characteristics, small size and ease of genetic manipulation, the fly offers an exceptional model system for studying physiological processes, affording increased sample sizes and a plethora of unique experimental subjects. Yet despite looking visually distinctive from humans, flies also present a powerful tool for understanding how our brains work, as many central nervous system pathways have been evolutionarily conserved between the two. Furthermore, research has demonstrated fruit flies exhibit behavioral responses, such as responding to drugs of abuse and exhibiting learning and memory, which are amazingly comparable to human behaviors. The overall goal of my thesis was to develop methods capable of quantifying neurochemicals within *Drosophila*. Chapters 2 focused on creating an updated set of CE-EC optimized separation conditions for the analysis of an increased number of biogenic amines in fruit fly heads. Chapters 3 and 4 utilized the separation method developed in Chapter 2 to investigate biogenic amines in dissected drosophila brains. In Chapter 3, hand dissection was utilized to investigate biogenic amine variations between genetic variants of fruit flies. Removal of the fly’s hard cuticle and eyes helped to measure biogenic amines differences within individual fly brains, even down to specific sections of the fly brain. In Chapter 4, a method of freeze-drying was described that makes the fly-head brittle
affording faster cuticle removal and increased sample sizes. Chapter 5 introduced CE-MS methodology to this work, adding the ability to verify the presence of biogenic amines within the fly, new analytes of interest including other neuromodulators and of applied (fed) drugs.

The Future of Fly Measurements

Measuring Biogenic Amines in Drosophila Using Online Sampling

Separations of chemical components from single and populations of dissected Drosophila brains provides a method that can be used to investigate the roles neuromodulators play within the brain, and dissected brains even offer the ability to obtain regionally specific information about the brain. Yet the dissection process imparts some variance to the samples; care must be taken to ensure samples contain only the region of interest while preserving the entirety of the neurons in that region. Additionally, these experiments require that the sample be dissolved in some small amount of solution, typically 1 µL, in order to inject it onto the capillary column. Given these two restrictions, online sampling techniques could be used as an approach to improve upon the methods presented here and investigate the extra- and even intra-cellar amounts of neuromodulators present within the fly.

To fashion an on-line sampling system for the analysis of Drosophila neurons, an etched microinjector can be fashioned on the injection end of the separation capillary
(Figure 6-1A). To do this, approximately 1 cm of the polyimide coating can be removed from the capillary exposing the fused silica. This end of the capillary would then be placed in hydrofluoric acid while being purged with 200 psi of He gas, which helps control the diffusion of hydrofluoric acid into the capillary. After 10 minutes, 2 mm of the exposed fused silica is removed from the hydrofluoric acid and left to etch for an addition 35 minutes. The capillary is then removed from the hydrofluoric acid, neutralized, and cut to length with a scalpel to remove any etched i.d. of the capillary.

**Figure 6-1** (a) SEM image along the length of a microinjector fabricated from the end of a 770-nm i.d. capillary. Scale bar represents 500 µm. (b) SEM image of the tip of a microinjector of a 770-nm i.d. capillary at a higher magnification. The etched silica at the end is only approximately 1 µm thick, resulting in an injector that is approximately 3 µm in diameter. Scale bar represents 1 µm. Reprinted from the American Chemical Society.
By slightly removing the capillary after 10 minutes of etching, a long taper with a sharp tip is formed on the end of the capillary that can easily be used to directly sample from the fly. Unlike in Chapter 3 where there is a delay between sample acquisition and injection as the sample must be sonicated and centrifuged, the microinjector end of the capillary could be quickly placed over a region of interest, such as within a drosophila brain slice, with a micromanipulator and an injection made. By controlling the i.d. of the capillary, the type of experiment can be managed. Since it is the outer diameter of the silica that is etched (which can be etched down to as little as a 1 µm thickness), the bulk of the size of the microinjector tip is dictated by the i.d. of the capillary (Figure 6-1B). Utilizing a larger i.d. (e.g. 15 um), the microinjector could sample from the extracellular space from a specific region of the brain such as the mushroom bodies, whereas a smaller i.d. (e.g. 5 µm) a target cluster of neurons. Using an even smaller i.d. capillary (1 µm to...
submicron), the capillary might be directed to sample from a particular neuronal junction or even inserted inside of a single cell to sample from the cytosol.

Chemical dynamics could be sampled from fly brains \textit{in vivo} by utilizing a fly collar, a 38.1 mm diameter concave plexiglass disk with a 1.0 mm hole in the center, such as the one developed by the Ewing group for \textit{in vivo} imaging and voltammetry.\textsuperscript{3} To insert a fly into the collar, the fly is first temporarily immobilized with ice, and its head is placed in the hole in the collar. Then low melt agarose is applied to the body and bottom portion of the head, making sure to leave the upper portion of the head uncovered. The top portion of the cuticle could then be removed using dissection scissors and forceps, exposing the fly’s brain. The microinjector could then be inserted into the fly head to make the injection. Flies have been found to be viable for upwards of 2.5 h. With separations lasting approximately 0.5 h, multiple experiments of a single area or even different areas within the fly could be explored with such a method.

**Investigating Alcohol Addiction in \textit{Drosophila}**

A major focus of developing separation methods from fly brains using MEKC-EC has been to study the effects that drugs of abuse, mainly the mechanisms underlying addiction and tolerance, has on biogenic amines.

The small chemical size and dual solubility of ethanol in both lipid media and water allow it to quickly enter the blood stream after consumption, pass through the blood brain barrier, and enter the brain. Once there it can begin to start interacting with many of the neurotransmitter systems present there. When alcohol enters the brain, it
primary acts on GABA_A receptors enhancing the effect of GABA, the major inhibitory neurotransmitter, release and results in an over-all inhibitory effect in the brain. It has also been shown that other neurotransmitter receptors, such as N-methyl-D-aspartate (NMDA) and serotonin 3 (5-HT_3) receptors, can be affected by alcohol. Alcohol can inhibit NMDA receptors, a receptor of the major inhibitory neurotransmitter glutamate, and further enhance the over-all inhibition seen within the brain by exciting other inhibitory, such as GABA releasing, neurons. It has also been shown that acute alcohol intake (a single episode) can directly potentiate 5-HT_3 receptor function, resulting in further downstream inhibitory effects. As alcohol exposure persists over days, the brain becomes tolerant and dependent upon the substance, such as in the mesolimbic pathway where alcohol, a rewarding stimuli, in the ventral tegmental area excites dopamine neurons that project into the nucleus accumbens, mediating the reinforcement process.

Preliminary studies have been conducted in conjunction with the Han lab in the Department of Biological Sciences at The University of Texas at El Paso and former Ewing group members Paula Powell, Tracy Paxon, and Imee Arcibal. These studies were aimed to examine the effect of acute and chronic exposure to alcohol on the biogenic amine levels of *Drosophila* with MEKC-EC. Utilizing behavioral observations in the Han lab, chronic tolerance develops in flies that have been exposed to large doses of alcohol for 7 days, and these flies remain sensitive to alcohol for three day thereafter. Three experimental groups were developed to study chemical changes in the brain during tolerance: a chronically exposed group which was exposed to intoxicating quantities of ethanol for 7 days, a single exposure group which was exposed to ethanol one time on
day, and a control group which was not exposed to ethanol. Additionally, two additional experimental groups were developed to study the effects of abstinence: an abstinent group that has been chronically exposed to alcohol for 7 days and then given 3 days to recover and an age-matched control. Population samples consisting of 40-50 heads per sample for all five groups have been run using MEKC method developed by Paxon and Powell\(^9\) and analyzed (Table 6-1). While no obvious trend is visible within the data collected, large variations in the results are apparent that are inconsistent which could result from variation within peak area analysis. One future proposal would be to use the updated separation method from Chapter 2 combined with the dissection methods from Chapters 3 and 4. The updated separation method uses a more tightly controlled buffer and might be better to investigate the alcohol related metabolites salsolinol and norsalsolinol. Given that salsolinol and norsalsolinol are synthesized by acetaldehyde and formaldehyde, metabolites of ethanol and methanol respectively, with dopamine,\(^{10,11}\) analysis of them within flies could offer a means of examining alcohol use and abuse. Reduction of the large overloading peaks with the better separation conditions developed should result in smaller peaks of interest which will be easier and more consistent to identify.

Several alcohol-related mutants have been developed, such as *cheapdate*,\(^{12}\) *tipsy*,\(^{13}\) *barfly*,\(^{13}\) and *hangover*.\(^{14}\) Both *cheapdate* and *tipsy* mutants express increased sensitivity to alcohol, *barfly* expresses reduced sensitivity to alcohol, and *hangover* expresses reduced development of tolerance. It would be interesting to compare the behavioral effects of alcohol on these mutant flies and a control group against changes in biogenic amine and metabolite levels measured by capillary electrophoresis. To do this,
mutant and control flies could be exposed to alcohol in an apparatus designed to measure the adaptive behavior associated with exposure to alcohol (the amount of time it takes for the flies to lose postural control) such as an fly inebriometer or Flypub. Populations of flies would then be dissected, sacrificed at 0, 12, and 24 h after the alcohol exposure, and analyzed using MEKC-EC. Dissection ensures a cleaner sample that that can be more consistently analyzed, while the different sacrifice times will help to compare the effects of the brain on alcohol versus various states of recovery. Similar to the experiments outlined earlier, flies exposed to alcohol for multiple days and thus chronically tolerant to alcohol could be studied. Not only would such experiments help to understand the role biogenic amine and metabolite levels play in addiction and tolerance, but they could also help to understand why certain genes make some people more prone to alcohol addiction.

Table 6-1 Compilation of quantified biogenic amine and metabolite amounts obtained from separations of populations of flies exposed to different amounts of ethanol.

<table>
<thead>
<tr>
<th>Analyte Amount (fmol)</th>
<th>Dopamine</th>
<th>I-Dopa</th>
<th>N-acetyl dopmaine</th>
<th>Octopamine</th>
<th>N-acetyl octopamine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chronic</td>
<td>32.5 ± 14.3</td>
<td>2130 ± 575</td>
<td>547 ± 126</td>
<td>275 ± 84.2</td>
<td>102 ± 29.8</td>
</tr>
<tr>
<td>Single Exposure</td>
<td>65.8 ± 16.6</td>
<td>4770 ± 1510</td>
<td>566 ± 77</td>
<td>311 ± 61.3</td>
<td>344 ± 143</td>
</tr>
<tr>
<td>Age-Matched Chronic</td>
<td>347 ± 247</td>
<td>6260 ± 2580</td>
<td>748 ± 294</td>
<td>1230 ± 502</td>
<td>321 ± 129</td>
</tr>
<tr>
<td>Abstinent</td>
<td>94.7 ± 22.8</td>
<td>3370 ± 579</td>
<td>540 ± 134</td>
<td>345 ± 98.2</td>
<td>210 ± 63.5</td>
</tr>
<tr>
<td>Age-matched Abstinent</td>
<td>187 ± 61.2</td>
<td>5150 ± 1100</td>
<td>1370 ± 284</td>
<td>89.9 ± 35.6</td>
<td>261 ± 71.8</td>
</tr>
</tbody>
</table>
References

VITA
Nicholas John Kuklinski

EDUCATION:

Ph.D. in Chemistry, December 2010, The Pennsylvania State University
B.S. in Chemistry and Statistics, Mathematics Minor, May 2003, North Carolina State University

PUBLICATIONS:


ORAL PRESENTATIONS:


