GÖTEBORGS UNIVERSITET

Static and Dynamic Measurement of Neurotransmitters in *Drosophila* Brain

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Static and Dynamic Measurement of Neurotransmitters in *Drosophila* Brain

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Cover picture: *Drosophila Melanogaster* genetically modified with green fluorescent protein tagged choline acetyltransferase localized to acetylcholine releasing cells. Inset, an electropherogram of electroactive species in a single homogenized brain.

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To *Drosophila melanogaster*, may your sacrifice not be in vain

ABSTRACT

Neurotransmitters, the substances neurons use for communication, and their precursors and metabolites are of obvious importance for the wellbeing of the individual and when the neurotransmitter balance is off it can lead to catastrophic suffering as in the addiction to drugs or in neurodegenerative diseases. By understanding how neurons communicate with the environment, treatment may be found to aid in the symptoms of unbalance. *Drosophila melanogaster*, the fruit fly, has been shown to be an excellent model for understanding neuronal processes and behaviors. Although the adult fly has a simpler nervous system than those of vertebrates, it is capable of higher-order brain functions, including aversive and appetitive learning, and recalling learned information from prior experiences. Invertebrate models, such as *Drosophila melanogaster* have been used previously to investigate neurochemical changes in the CNS associated with drug addiction as well as in the of study of neurodegenerative diseases such as Alzheimer's disease, Huntington's disease, and Parkinson's disease by *Drosophila* mutants. Many of the neurotransmitters associated with these diseases occur in minute amounts and can be difficult to detect in the small volume of the fly brain. As such, it is essential to develop analytical tools for these unique biological systems that can be quickley performed and accurately analyses the neuronal substances as well as requiring extremely small sample volume. Capillary electrophoresis and *in vivo* voltammetry are two methods that meet these requirements.

In Paper I a new separation scheme for capillary electrophoresis was devised to allow resolution of 23 neurotransmitters, metabolites, and precursors. In fly homogenates a focus on six of the substances thought to be involved in the response to alcohol were identified. In Paper II the removal of the cuticles and eyes leaving only the brains further enhanced the separation profile of neurotransmitters from Paper I. In Paper III a method for sample preparation by freeze drying the Drosophila brains was presented. The use of freeze-dried samples offers a way to preserve the biological sample while making dissection of the tiny brain samples easier and faster. This provides more concentrated samples and with that higher signals and better detection limits. In Paper IV the effect of cocaine on the dopamine transporter was shown to be reduced by the ADHD drug methylphenidate using in vivo voltammetry.

LIST OF PUBLICATIONS

This thesis is based on the following papers listed. They are appended at the end of the thesis and will be referred to in the text by their roman numerals.

Paper I Kuklinski, N. J., **Berglund, E. C.,** Engelbreksson, J., and Ewing, A. G. (2010) "Determination of Salsolinol, Norsalsolinol, and Twenty-One Biogenic Amines Using Micellar Electrokinetic Capillary Chromatography-Electrochemical Detection", *Electrophoresis 31*, 1886-1893.

Paper II Kuklinski, N. J., **Berglund, E. C.,** Engelbrektsson, J., and Ewing, A. G. (2010) "Biogenic Amines in Microdissected Brain Regions of *Drosophila melanogaster* Measured with Micellar Electrokinetic Capillary Chromatography-Electrochemical Detection", *Anal. Chem. 82*, 7729-7735.

Paper III **Berglund, E. C.,** Kuklinski, N. J., Karagündüz, E., Ucar, K., Hanrieder, K., and Ewing, A. G. "Freeze-Drying as Sample Preparation for Micellar Electrokinetic Capillary Chromatography – Electrochemical Separations of Neurochemicals in *Drosophila* Brains", s*ubmitted to ACS Analytical Chemistry.*

Paper IV **Berglund, E. C.,** Makos, M. A., Keighron, J. D., Phan, N., Heien, M. L., and Ewing, A. G. E "Oral Administration of Methylphenidate Blocks the Effect of Cocaine on Uptake at the *Drosophila* Dopamine Transporter," *submitted to ACS Chemical Neuroscience.*

RELATED PAPERS

Paper V Makos, M. A., Kuklinski, N. J., **Berglund, E. C.,** Heien, M. L., and Ewing, A. G. (2009) "Chemical Measurements in *Drosophila"*, Trends Analyt Chem 28, 1223-1234.

Paper VI Kuklinski, N. J., **Berglund, E. C.,** and Ewing, A. G. (2010) "Micellar Capillary Electrophoresis – Electrochemical Detection of Neurochemicals from *Drosophila"*, J Sep Sci 33, 388-393.

Paper VII Trouillon, T., Svensson, M. I., **Berglund, E. C**., Cans, A-S., and Ewing, A. G. (2012) "Highlights of selected recent electrochemical measurements in living systems" Electrochimica Acta, in print and available online 18 June at http://dx.doi.org/10.1016/j.electacta.2012.04.164.

CONTRIBUTION REPORT

There are multiple authors on the papers presented here and my contribution to each of them is listed below.

Paper I — I was involved in the planning and conducting of the project with Nick Kuklinski. I was part of the data analysis, figure preparation and wrote parts of the manuscript.

Paper II — I was involved in the planning and conducting of the project with Nick Kuklinski. I had a minor part of the data analysis and figure preparation and wrote parts of the manuscript.

Paper III — I planned the final project, coordinated the experimental aspects of the project between several people, and was responsible for the data analysis with help from Jörg Hanrieder, for the figures and the final manuscript.

Paper IV — I planned the final project, conducted most of the experiments and the data analysis, made all the figures and wrote a major part of the manuscript.

TABLE OF CONTENTS

ABBREVIATIONS

Abbreviations commonly used in this thesis:

ADHD	Attention deficit hyperactivity disorder
CE	Capillary electrophoresis
ChA-GFP	Drosophila mutant carrying choline acetyltransferase
CNS	Central nervous system
CZE	Capillary zone electrophoresis
EOF	Electroosmotic flow
EPM	Electrophoretic mobility
fmn	Drosophila mutant with knocked dopamine transporter
FSCV	Fast scan cyclic voltammetry
GABA	Gamma aminobutyric acid
GFP	Green fluorescent protein
i.d	Inner diameter
L-DOPA	L-3,4-dihydroxyphenylalanine
MEKC	Micellar electrokinetic capillary chromatography
PNMT	Phenylethanolamine N-methyltransferase
SDS	Sodium dodecyl sulfate
TH-GFP	Drosophila mutant carrying tyrosine hydroxylase
white	Drosophila mutant with unpigmented eyes

1 BASIS OF NEUROTRANSMISSION

1.1 Introduction

"All living things are made of cells" (*1*). Cells are essentials to life. In a matter of fact cells are life and without cells there is no life. Cells can be found in various shapes and forms and all have their designed task in the machinery of life. In an organism, they are not isolated; they are in constant contact with their environment, foremost with other cells. When cell-to-cell communication is of balance the functions the cells control and regulate will be affected and the previous well being of the individual will disappear. Many drugs of abuse affect cell-to-cell communication and many of the neurological diseases originate from a disturbance in communication between nerve cells. Some of these diseases are touched on at the end of this chapter. It is important to understand the sensitive balance of cell-to-cell communication to be able to treat it when it is off balance. The work in this thesis contributes to the understanding of which molecules nerve cells use to communicate with their environment, both by release and re-uptake of the molecules and in the case of re-uptake, a special focus will be on the neurotransmitter dopamine and its transporter.

1.2 The Cell

Before going into how cells communicate in any detail it is important to have an understanding of what makes up these cells. There are two types of cells, the smaller and simpler prokaryotes and the more complex eukaryotes. The prokaryotes are divided into two groups, the bacteria and the archaea and live mostly as single celled organisms (*1*). The eukaryotes are divided up into animal, plant and fungal cells and mostly form multicellular organisms (*1*). The main deference in constitution between these cell types is that eukaryotic cells contain a cell nucleus and membrane-bound compartments wherein the intracellular components are confined. In prokaryotes the intracellular components are only confined by the plasma membrane. In both cases the plasma membrane keeps the intracellular part of the cell separated from the surrounding environment. This membrane is like a fluid double layer of lipids packed, along with some proteins, making the membrane mostly impermeable to water. Outside the plasma membrane is the extracellular matrix with a network of polysaccharides and proteins. The inside of the cell contains the fluid cytoplasm and the cells' compartments, the organelles.

1.3 Cell communication

There are various ways a cell can communicate with another cell. If the cells are close enough for the plasma membranes to make contact communication can be achieved by contact-dependent signaling where the signal molecule is bound to the membrane and exposed to the extracellular matrix. The target cell has a receptor, a protein that specifically binds to the signal molecules unique 3D structure attached to its membrane and when the two cells make contact the signal molecule binds to the receptor and a response is evoked in the target cell. This can also be achieved without the receptors. Then the two cells in contact form a gap junction (a small hole between them) and share small signal molecules though the directly connected cytoplasm. If the cells are close but not so close to make contact, diffusion through the plasma membrane into the local environment will occur, this is called paracrine signaling. For this type of signaling it is important the target cell is close and that the receptor has a high affinity towards the signal molecule. The most common signaling pathway though is release from the signaling cell to the target cell. Another cell type that use release is the endocrine cell, is a specialized signaling cell that controls the behavior of the organisms as whole. It releases its signaling molecules out into the blood stream where they are carried to target cells throughout the body. This type of signaling is used for longer distances in different parts of the body. Another type of cell that uses release and signals over great distances is the cell type called a neuron or nerve cell and it is this type of cell and its signaling chemicals, the neurotransmitters, which will be in focus for this work. (*1*)

1.4 Neurons

The purpose of the neuron is to receive, conduct and transmit signals and it does that in a complex network that constitutes the nervous system. The neuron (Figure 1) is composed of three parts, a cell body which contains the nucleus, the dendrites, and the axon. Around the cell body a network of dendrites branches out like a star around the nucleus. The branching of the dendrites acts as an antenna and receives chemical signals over a great surface area. It can receive as many as 100,000 inputs in a single neuron (*1*). The chemical signal is transformed to an electrical signal and is passed on to the axon, the elongated structure protruding out from the cell body with a length ranging from less than a mm up to more than 1 m (*1*) for communication over long distances. At the terminal of the axon it divides into branches for communication at multiple sites.

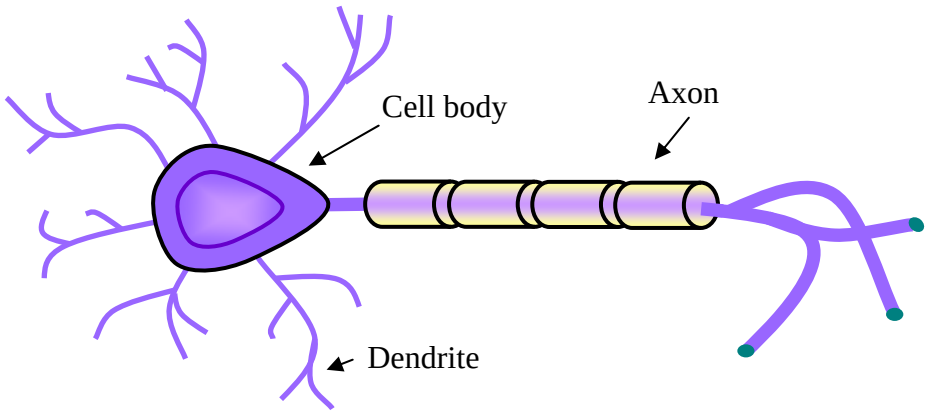


Figure 1. The neuron is composed of three parts, a cell body which contains the nucleus, the dendrites, and the axon.

1.5 Synaptic transmission

The idealized cell-to-cell communication (Figure 2) between two neurons is thought to consist of a dendrite and an axon terminal communicating by sharing neurotransmitters over short distances, but the interactions can also be axon-to-cell body, axon-to-axon and dendrite-to-dendrite. The cells communicating form a synaptic cleft where the neurotransmitters are released from the axon and recognized by the receptors on the dendrite. The binding of neurotransmitters to the receptors begins the building up of an action potential at the dendrite that travels to the cell body and once accumulated along the axon. At the axon side of the synaptic cleft the potential causes the opening of voltage-gated ion channels allowing ions to flow into the cell until the change of potential, depolarization, over the membrane has opened many ion channels. The influx of the ions initiates the process of exocytosis or release of neurotransmitter. After a short time the potential is reversed and the channels close again and release is stopped. Exocytosis is the release of the neurotransmitters from the synaptic vesicles from the axon into the synaptic cleft. These neurotransmitters diffuse to and bind the receptors on the receiving neuron and thereby activate (or inhibit) the receptors causing responses such as new action potentials to be initiated in the post-synaptic cell. The neurotransmitter bound to the receptors is released after a short time and is accumulated again into the pre-synaptic cell again, and some is apparently repacked into the vesicles again. (*1*)

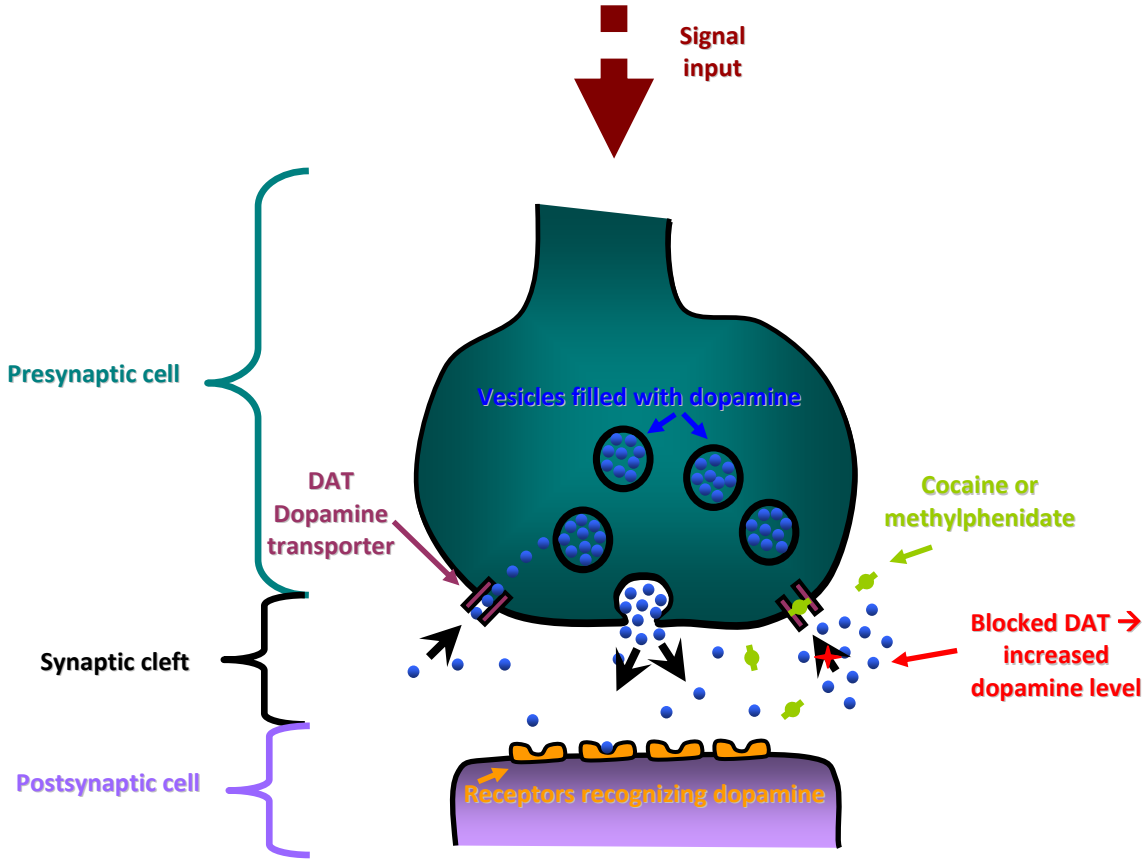


Figure 2. The idealized cell-to-cell communication between two dopamine neurons. The dopamine transporter is blocked here with the drugs cocaine or methylphenidate

1.6 Amplification of the action potential

The action potential can only travel so far and so fast without amplification. For the action potential to travel with greater speed the axon is covered with hard packed segments of insulating myelin sheaths that are made from the supporting glial cells. The sheets are typically 1 mm in length and the gap between them, the nodes of Ranvier, are about ~1 μm (*2*). Most of the axons voltage-gated ion channels are concentrated in the nodes of Ranvier. When one node feels the effect of the action potential the membrane potential changes (depolarization) and the voltage channels open and the ions flows in to the cell. Since the sheathed segments of the axon have cable-like properties, a depolarization at one node almost immediately spreads to the next one. This amplifies and speeds up the signal. If the neuron is a part of the peripheral nervous system then the glial cells responsible for myelination are called Schwann cells and if the central nervous system the glial cells are oligodendrocytes.

1.7 Neurotransmitters

Neurotransmitters are the chemicals that neurons use for communication with other cells in its environment. It is hard to define what a neurotransmitter is since the definition of this term has changed over the years as new types of molecules have been discovered to be neurotransmitters. The traditional criteria for a molecule to be called a neurotransmitter include that the neurotransmitter is a compound that must be synthesized and released pre-synaptically. The release must originate from depolarization and when released, it must act on a post-synaptic receptor and elicit a biological response. If the compound is applied post-synaptically it should have the same effect as when it is released by a neuron. After the release of the compound the action of it must be inactivated either by reuptake or by enzymatic activity. However, not all of the neurotransmitters of today meet all the above criteria. Some say they should not be called neurotransmitters then but since it is accepted practice they often are.

There are two basic effects the neurotransmitters can give rise to when released from a neuron; they can either be excitatory or inhibitory. The excitatory neurotransmitters pass on the response leading to an increase in the probability that the neuron will fire an action potential. The inhibitory transmitters decrease the probability of new fired action potential.

Table 1. Classification of neurotransmitters according to their chemical group. Modified from (*3*)

	Chemical group	Examples
A	**Choline ester**	Acetylcholine
B	**Monoamines**	
	Catechol	Dopamine, Norepinephrine
	Indole	Setotonin
	Imidazole	Histamine
C	**Amino acids**	
	Acidic	Glutamate
	Basic	GABA, Glycine
D	**Peptides**	Enkephalins, Endorphins, Cholecytokinin
E	**Purines**	Adenosine triphosphate (ATP), Adenosine
F	**Steroids**	Pregnenalone
G	**Nitric oxide**	
H	**Eicosanoids**	Prostaglandins

Neurotransmitters can be classified based on their chemical structure. In *Neurotransmitters, Drugs and Brain Function* edited by Roy Webster 2001 (*3*) they list five groups (A-E in Table 1) that are considered to be true neurotransmitters and three that are not so clear. The first three classes are the most common ones. This work includes mostly the monoamines.

1.8 History of and diseases associated with neurotransmitters

1.8.1 Acetylcholine

In 1921 Otto Loewi from Germany discovered the chemical transmission of nerve impulses. He studied how vital organs responded to chemical and electrical stimulation and he called the first isolated neurotransmitter "Vagusstoff" since it was isolated from the vagus nerve. Later "Vagusstoff" was given the name acetylcholine. This transmitter can be found both in the peripheral nervous system (PNS) and central nervous system (CNS) as well as in the autonomic nervous system (ANS). Acetylcholine can be either excitatory or inhibitory but most often it works as an excitatory transmitter (*1*). It is used by the motor neurons of the spinal cord and is therefore released at all vertebrate neuromuscular junctions (*4*). It affects the cardiovascular system by decreasing the cardiac rate. In the gastrointestinal system it increases peristalsis in the stomach and it also affects the respiratory system and the urinary tract. In botulism, the poisoning by ingested botulinum toxin (often from improperly sterilized food being in contact with soil) damages the autonomic nervous system by blocking the release of acetylcholine in the fine nerve fibrils responsible for muscles contraction. This will result in paralysis. There is a link between acetylcholine and people suffering from Alzheimer's; there is on the order of a 90 % loss of acetylcholine in the brain of people with Alzheimer's disease.

1.8.2 Norepinephrine

The second neurotransmitter to be discovered was norepinephrine by the Swedish scientist Ulf von Euler in 1946. He both identified norepinephrine as well as that it is stored within nerve fibers themselves. Norepinephrine, also called noradrenalin, is both a hormone as well as a neurotransmitter and is involved in alertness and arousal, and has influences on the reward system. It has been proposed that norepinephrine may also be associated with depression (*4*).

1.8.3 Epinephrine

Epinephrine, also called adrenaline, is also a hormone and neurotransmitter like norepinephrine. Epinephrine acts on nearly all body tissues and is synthesized via methylation of norepinephrine by phenylethanolamine N-methyltransferase (PNMT). It was first isolated by the Polish physiologist Napoleon Cybulski in 1895 but its roll as neurotransmitter was not clear until 1982 (*5*). Interestingly norepinephrine must be synthesized in the vesicles and released out to the cytosol of adrenergic axonal terminals for the synthesis of epinephrine (because PNMT is located there) and then transported into vesicles for release (*6*). Epinephrine regulates heart rate, blood vessel dialation and air passage and is released as major a component of the fight-or-flight response. Epinephrine is also used as a drug to treat cardiac arrest and can also be used as a bronchodilator for asthma.

1.8.4 Dopamine

The neurotransmitter dopamine was discovered as a neurotransmitter in the 1950s by Arvid Carlsson when he demonstrated that it was more than just precursor for norepinephrine. Dopamine is an inhibitory neurotransmitter and is strongly associated with the function of the brain, involved in the reward system as well as the control of movement. Drugs of abuse such as alcohol, cocaine and heroin increase the levels of extracellular dopamine and are responsible for the euphoria associated with these drugs. Excessively high levels of dopamine are not good. The mental illness schizophrenia has also been shown to involve excessive amounts of dopamine (*7*). Low levels of dopamine have been shown to have negative effects too. The lower levels of dopamine in the brain structure basal ganglia, a structure in the base of the forebrain of the brain, are responsible for the uncontrollable muscle tremors in Parkinson's syndrome. The cause is degeneration of dopamine neurons. Arvid Carlsson found out that the precursor L-dopa could be used to elevate the dopamine levels Parkinson's and has been used as a treatment since then.

1.8.5 GABA

Another inhibitory neurotransmitter is the GABA (gamma aminobutyric acid) discovered to be a part of the CNS in the 1950s even though it was first synthesized in 1883. It is found in the CNS of mammals in high concentration (*8*). Since GABA is an inhibitory neurotransmitter it hinders the transmission from one cell to another and thereby has a quieting influence. Without GABA the neurons would fire too

often. A low GABA level has been associated with anxiety disorders and also with Parkinson's syndrome.

1.8.6 Serotonin

Serotonin was discovered in the 1930s by Vittorio Erspamer and in 1948 if was found in blood serum by Irvine Page. Page named it serotonin from "serum-tonic". Later it was also proven to be an excitatory neurotransmitter. Serotonin has been found to have a key role in the regulation of development, mood, sleep and behavior. Serotonin also has some cognitive functions including memory and learning and regulating attention. Even though it is important in the brain most of the serotonin can be found in the digestive system. A change in the serotonin balance has a huge impact of the well being of the individual. A lower level than normal has been shown to lead to depression, problems with anger control, obsessive-compulsive disorder, and suicide. Too little also leads to an increased appetite for carbohydrates (starchy foods) and trouble sleeping, and is also associated with depression and other emotional disorders. It has also been tied to migraines, irritable bowel syndrome, and fibromyalgia.

1.8.7 Tyramine and octopamine

Tyramine and octopamine are the invertebrate's counterparts of the vertebrate's epinephrine and norepinephrine and were discovered as neurotransmitters in the 1950's (*9, 10*), in 1948 Octopamine was discovered by Vittorio Erspamer, the same person responsible of serotonin. Octopamine was named for the *Octopus* where it was first extracted from the salivary glands (*11*). Tyramine and octopamine regulate the fight or flight response, motivation, and aggression in invertebrates (*11*).

1.9 Aim

The aims of this thesis work have been to improve the separation and quantification of neurotransmitters in adult *Drosophila melanogaster* and go a more deeply explore how dopamine and its transporter are affected by the psychoactive drug cocaine and the attention deficit hyperactivity disorder (ADHD) drug methylphenidate.

In P**aper I** an existing protocol used to separate neurotransmitters in fly head homogenate was optimized to be able to separate a standard of 23 neurotransmitters and its metabolites and precursors. In fly homogenates a focus on six of the

substances thought to be involved in the response to alcohol were identified, dopamine, salsolinol, norsalsolinol, N-acetyloctopamine, octopamine, and N-acetyldopamine. To our knowledge this is the first detection of salsolinol and norsalsolinol in the fly model. In **Paper II** it was shown that a matrix effect, which interferes with neurotransmitter quantification, mainly originates from the eye pigment and a better quantification method could be achieved by dissecting the brain out before separation. Also the amount of dopamine and octopamine from different brain regions was determined. In **Paper III** a method was presented to speed up dissection and also to increase the sample concentration by freeze drying the fly brain prior to analysis. In **Paper IV** the effect of cocaine on the dopamine transporter was shown to be reduced by the ADHD drug methylphenidate.

2 THE FLY MODEL IN NEUROSCIENCE

2.1 Introduction

As has been shown in the previous chapter neurotransmission is an important process in the nervous system and when it does not work properly it leads to catastrophic malfunctions for the individual. The process has been regarded as so important that previous mentioned Otto Loewi, Ulf von Euler, and Arvid Carlsson all received the Nobel Prize for their contributions in the understanding of neurotransmission (*12*) and the recent Nobel Prize in chemistry given for the G-Protein coupled receptors shows it is still regarded as an important field. In these types of studies typical experiments are carried out in model organisms. For example Otto Loewi dissected out two beating hearts from frogs and stimulated one heart to beat slower. Then he took fluid from the stimulated heart and applied it on the second one and saw the heart beat of the second unstimulated heart slowed down too in response to the fluid. This response was later found to result from the transmitter, acetylcholine, in the fluid. This type of experiment cannot be carried out in humans; thus there is a need for model organisms. A model organism is a simpler organism that can be used to increase knowledge about fundamental biological processes in a more complex organism. Some of the traditional model organisms include the *Escherichia coli* (bacteria), *Saccharomyces cerevisiae* (budding yeast), *Caenorhabditis elegans* (nematode worm), *Drosophila melanogaster* (fruit fly), *Arabidopsis thaliana* (mustard plant), *Danio rerio* (zebra fish) and the previous mentioned frog *Xenopus laevis (13)*. *Drosophila melanogaster* has been used as a model organism since the beginning of the last century when it first was used for genetic studies by Thomas H. Morgan. Research using *Drosophila* has led to important insights into the mechanisms of human developmental and physiological processes and has resulted in many Nobel Prizes with the first to Thomas H. Morgan in 1933 (*12*).

2.2 Validating the fly model

The *Drosophila* fly is biologically simpler than the human with only 4 chromosomes and ~14 000 genes (*14*) and a volume of the brain of ~0.002 mm^3 compared to the human brain of ~1200000 mm^3, thus making the fly model an interesting, but challenging small model to use in neuroscience. Despite its small size, it has been shown that the adult fly is capable of higher-order brain functions including aversive and appetitive learning and recalling learned information from previous experiences

(*15, 16*) as well as an ability to display anthropomorphic behavior aggression (*17*). The larvae can be used to investigate basic neurotransmission and chemosensory pathways (*18*) as well as the fundamental aspects of glial biology (*19*). The conservation between the *Drosophila* and mammalian proteomes is high with approximately 50 % of the protein sequence in the fly having similar counterparts in the human (*20*) and 714 distinct human disease genes have been matched with 548 unique *Drosophila* sequences, 74 of these genes are categorized as neurological (*21*). Neurotransmitters such as dopamine, serotonin and tyramine are known to be involved in physiological processes found in both mammalian and *Drosophila* systems (*16, 22-25*) and for the vertebrate specific neurotransmitters epinephrine and norepinephrine the analogues tyramine and octopamine are thought to have similar physiological roles (*11*). Dopamine has for example been associated in human and fly behavior as reward and motivation, sleep cycles, alcohol tolerance and sensitivity to addictive drugs (*22, 23, 26*).

The *Drosophila* fly has been used in genetic studies as well as research for developmental biology for over a century and a great deal of the genetics of *Drosophila* has been explored. A comprehensive database of *Drosophila* genetics and genomics can be found at http://flybase.org (*27*). Furthermore, the *Drosophila* genome contains fewer genetic redundancies compared to vertebrates which has facilitated identification of individual genes and molecules involved in particular behaviors (*28*). These facts are the reason it has been relatively straightforward to get the feature/behavior you want in the *Drosophila* model via genetic manipulation and even more so after the genome of *Drosophila* was fully sequenced (*14*). Complex behavioral patterns found in mammalian system with regard to learning and memory, courtship, alcohol tolerance, and circadian rhythms have been studied with *Drosophila* using genetic mutants (*29-32*). At Bloomington *Drosophila* Stock Center (*33*) there are more than 50 000 stocks of flies listed and 665 of these fly stocks are related to human diseases; 140 of these are mutants for the study of different processes of the neurodegenerative disease Alzheimer's. All of the above facts make the *Drosophila* fly a highly competent model to elucidate the roles of transmitters in human behavior as well as in neurodegenerative diseases.

2.3 *Drosophila melanogaster*

The red-eyed amber color fruit fly thrives in fermented vegetables or fruit and is common in the household in fall when fruit tends to ferment more. The adult fly does not eat much but the larva is eating constantly so the female fly is drawn to the

fruit to lay their eggs, the males are drawn to the fruit to find females. The fly is ~3 mm in length, the female slightly bigger than the male, see Figure 3. The male can be distinguished from the female by its size, but this can be misleading when determining sex because the flies are bigger in size at the beginning of their adult phase. In order to sex the flies it is more reliable to look at the color at the end of the abdomen, the reproduction parts but this can also be misleading the first couple of hours after emerging from the pupal. The abdomen is striped for both male and female but for the male it ends with a darker part, see Figure 3. Also the males have a black spot on their front legs; the sex combs important for male to be successful in copulation (*34*). The sex combs can be a bit tricky to see without a microscope but it is not impossible and under a microscope it is by far the safest way to determining the sex of the fly since the combs does not change during the adult life time.

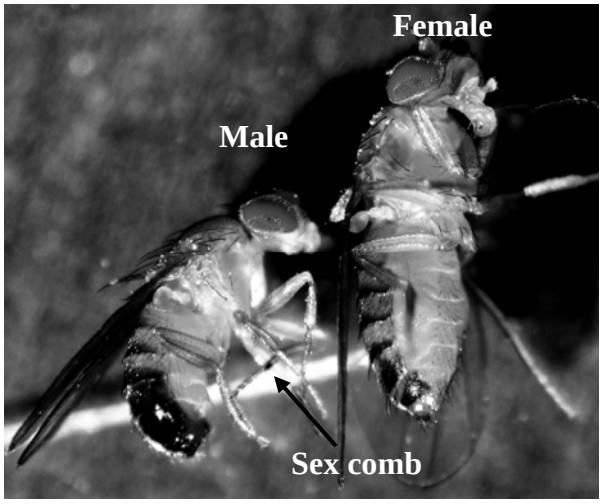


Figure 3. Male to the left, female to the right. The abdomen is striped for both male and female but for the male it ends with a darker part.

Females are ready for copulation at 8-12 h after they are hatched but the males do not mate efficiently until after three days (*35*). When the female *Drosophila* is ready she produces pheromones on her external cuticle. These pheromones are to attract the males but also for the male to be able to distinguish the sex and species of the female. The attracted male starts courting by sidling up to the female and tap the female's abdomen with his foreleg. He then extends and vibrates one of the wings laterally to produce a courtship song with bouts of 30 s to 1 min. The female use the song to recognize the species of the male. If the female does not kick the male or run away he will continue by licking the female's genitals before the attempt to mount her by curling his abdomen and beginning the coupling (*36*). This whole process involves

neurons in the male that projects from the parts of the mouth to the antenna lobes, from the antenna lobes to the lateral protocerebrum and to the mushroom bodies. The female can mate once or twice and store the sperms until the eggs need to be fertilized. The female then lays up to 100 eggs per day that hatch after half a day in a 25° C climate. Each larva goes through three stages over the next 4-5 days while constantly eating. To begin the next stage the larva migrate up the wall from the food and starts the formation of the pupa and stays in the pupa for a week until the adult fly emerges. The adult fly can live up to 14 days.

2.4 The brain of *Drosophila melanogaster*

The central nervous system of *Drosophila melanogaster* constitutes two parts, the brain and the ventral nerve cord (*37*). In the larvae the ventral nerve cord dominates the CNS (Figure 4B) and for the adult fly it is the brain (Figure 4A). In the larvae CNS the optic lobes and the brain make up the supraesophageal ganglion. By removing the supraesophageal ganglion the ventral nerve cord will be exposed (Figure 5A). Along the ventral nerve cord is the midline marked with a dashed line and inside, on either side of the midline are the neuropils (synaptically dense regions) containing dopamine and serotonin terminals (*38, 39*).

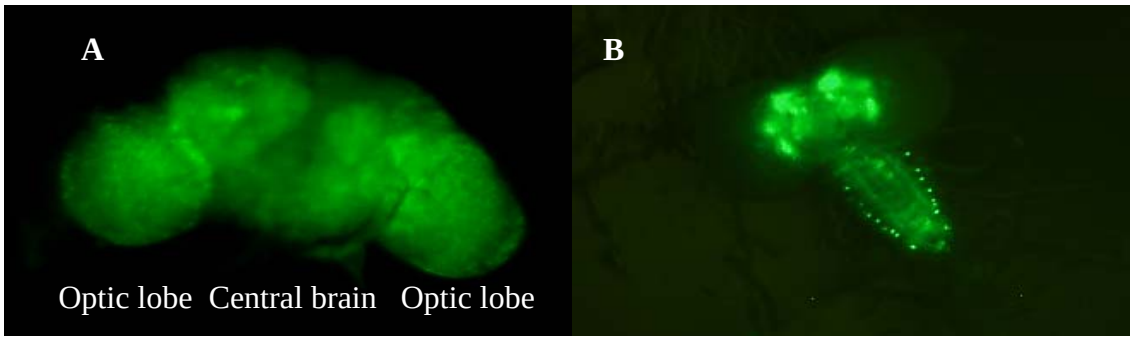


Figure 4. A) Brain from modified adult fly with green fluorescent protein tagged to choline acetyltransferase (ChA-GFP) to visualize acetylcholine releasing cells and **B)** modified larva CNS with green fluorescent protein tagged to tyrosine hydroxylase (TH-GFP) to visualize dopamine releasing cells.

On the outside of the ventral nerve cord are the motorneuron connections that protrude out to the rest of the larval body to the neuromuscular junctions where the action potential causes the muscles to contract. For the adult fly the CNS is distinctly different from that of the larva. The brain consists of two parts, the central brain, and the two optic lobes that receive inputs from the eyes. An interesting neuropil area in the adult fly is the mushroom body, which consists of two pared structures (see the blue structure in Figure 5B). (In the larva the mushroom body is a part of the supraesophageal ganglion). The ~2500 Kenyon cells (*28*) around each calyx project

through the calyx and the predunculus and bifurcate into the vertical (V) lobes or the horizontal (H) lobes. The vertical lobes contains the two subunits α and α' and the horizontal lobes the three β, β' and γ. There are three distinct clusters around the mushroom body that contain dopamine neurons, the protocerebral anterior median (PAM), protocerebral posterior lateral 1 (PPL1) and 2ab (PPL2ab) clusters. They project into and terminate in the mushroom bodies too. The PAM neurons project into the medial portion of the horizontal lob in the mushroom body, PPL1 neurons project to the vertical lobes, the junction area, the heel and distal peduncle, and PPL2ab neurons project to the calyx (*40*).

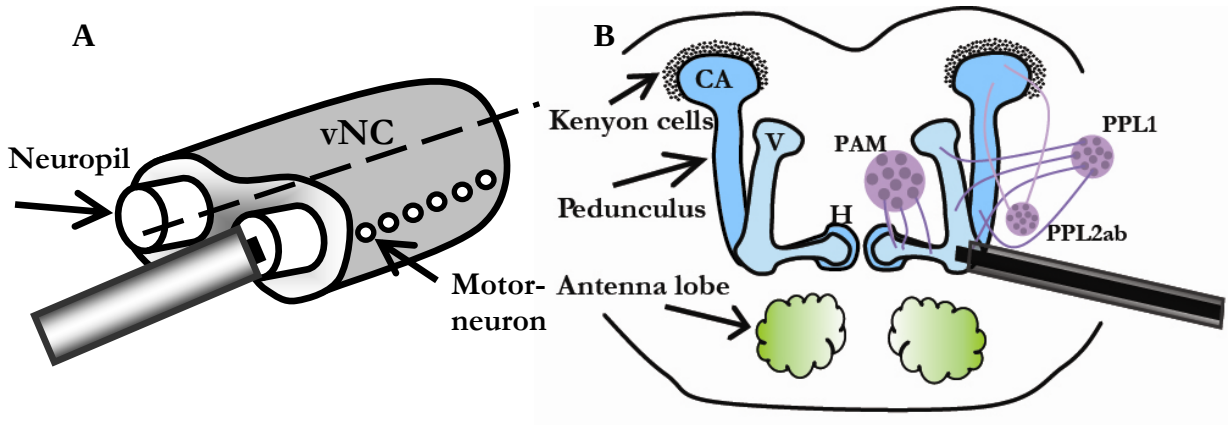


Figure 5. A carbon fiber microelectrode in **A)** ventral nerve cord (vNC) of larva with exposed neuropil. Along the ventral nerve cord is the midline marked with a dashed line. And **B)** adult fly brain with the mushroom body in blue and dopamine neurons in purple.

2.5 Mutations

The genetic modified *Drosophila* fly has been most valuable for this work but a mutant is of no value if it has nothing to compare to. As a control of the mutations effect the wild type fly is used. The wild type fly is a fly free from mutations. The cultures with the flies are isolated from its environment and the genetic pool in the containers is uniform after many generations side by side. Some of the classical wild-type stains are Canton-S, Oregon R-C, Oregon R-S, Berlin-K, and wild-type Berlin. The classical strains have changed over the years in captivity. The differences in olfactory preference have been studied where the classical wild-type has been compared to recently established wild-type strains and the study showed that the older strains had evolved an adaptive selectivity (*41*). This shows that one wild-type may not be equal to another and stress the importance to specify which wild-type used. In **Paper I-IV** the wild-type Canton-S was used.

2.5.1 TH-GFP

In **Paper IV** the mutant TH-GFP was also used to visualize the dopamine neurons. TH-GFP are transgenic flies carrying tyrosine hydroxylase (TH)-GAL4 and UAS-mCD::GFP (membrane tethered green fluorescent protein (GFP)). Tyrosine hydroxylase is the protein responsible for the hydroxylation of tyrosine to L-3,4-dihydroxyphenylalanine (L-DOPA), the biosynthetic precursor of dopamine. Since tyrosine hydroxylase is the rate-limiting step in dopamine biosynthesis (*42*), dopamine neurons should be in the same area of tyrosine hydroxylase. The system that allows for this visualization is the GAL4/UAS system by Brand and Perrimon in 1993 (*43*). GAL4 is a gene that encodes for the yeast transcription activator protein Gal4. The TH-GAL4 driver line produces flies with GAL4 only in neurons where tyrosine hydroxylase is present (*42*). GAL4 remains inactive in the fly until it binds an UAS responder line. The UAS can be made so it contains the protein green fluorescent protein (GFP). For the TH-GFP flies the UAS-GFP responder line had been crossed with the TH-GAL4 driver line to produce flies with GFP transcription in their TH-containing neurons. The TH-GFP fly was also used in **Paper II** where the neurotransmitter content was shown to correspond to the contents of the wild type flies.

2.5.2 White

Also in **Paper II** one of the oldest mutants known, the *white* mutant (*44*) was used. The *white* mutant has white eyes and was discovered as early as 1910 by Morgan. The *white* mutant strain contains a mutated copy of the w gene yielding unpigmented eyes.

2.5.3 *fmn*

For the experiments in **Paper IV** where the dopamine transporter was knocked out the hyperactive mutant fumin (*fmn*; meaning sleepless in Japanese (*23*)) that has a genetic lesion abolishing the dopamine transporter function was used. The genetic background of the *w;fmn* (the *fmn* mutation was backcrossed with *white* flies for a several of generations to recombinationally separate it from other lesions) mutant was replaced with the Canton-S background.

2.5.4 ChA-GFP

For visualization of the *Drosophila* brain the mutant ChA-GFP (Choline acetyltransferase with a GFP tag) was used. Choline acetyltransferase catalyzes the reversible synthesis of acetylcholine from acetyl coenzyme A and choline at cholinergic synapses. Acetylcholine is a major excitatory neurotransmitter in the central nervous system of insects (*45*) and is present all over the brain as can be seen in Cover Figure and Figure 4.

3 METHODS - small volumes

The central nervous system in the fly is 1/300 000 the size of the popular rat model making the fly a challenging system to use for *in vivo* methods in neuroscience. Its smaller size stresses the need for small volume analysis tools. A method that can be used to handle small sample sizes is capillary electrophoresis (CE). In CE ions migrate in an electrolyte solution under the influence of an electric field. Cations are attracted to the negative side (the cathode), anions to the positive side (anode). Since the ions have different mobilities they migrate through the capillary at different velocities. CE has been used in **Paper I** for separation of whole head homogenates, in **Paper II** for separation of dissected brains and brain regions, and in **Paper III** CE was used to separate freeze-dried brains where an improved sample preparation method was created.

3.1 Brief introduction to CE

In 1930 Arne Tiselius presented his thesis (*46*) on how to use electrophoresis as an analytical technique to separate blood plasma proteins, and in 1948 he received the Nobel Prize *"for his research on electrophoresis and adsorption analysis, especially for his discoveries concerning the complex nature of the serum proteins" (12)*. One of Tiselius' students, Stellan Hjertén, continued Tiselius work by developing the use of rotating quartz capillaries presented, which was presented in his thesis in 1967 (*47*). He called this free zone electrophoresis (*48*). The technique evolved and in 1979 Mikkers et al. (*49*) presented zone electrophoresis in Teflon tubes with 200-μm inner diameter (i.d). Though, it was not until 1981 that the technique became popular when Jorgenson and Lukacs really broke the barrier to highly efficient separations (less than micrometer plate heights) in electrophoresis (*50*) by the use of even smaller silica capillaries and the result is the capillary electrophoresis used today.

Capillary electrophoresis generally uses fused-silica capillaries with small i.d. commonly in the range from a few μm up to 100 μm and 10 to 100 cm in length. Sample volumes are small with low detection limits, and single cell analysis of enzymes with limit of detection as low as zeptomoles can be accomplished (*51*). The small size of the capillaries has enabled sampling of cytoplasm from inside large single nerve cells (*52*), separation of the contents in homogenates of single cells (*53*) and whole cells lysed inside the capillary prior separation (*54*). Even single vesicles with volumes as small as 65 aL have been lysed inside the capillary and separated (*55*).

CE separation of amino acids in small volume samples of *Drosophila* hemolymph (analogous to blood) from individual *Drosophila* larvae (*62*) and individual adults (*63*) have been performed, as well as separation of *Drosophila* RNA (*64*). As part of the scope of this thesis, the CE separation of neurotransmitters from *Drosophila* CNS (*56-61*) was examined.

Today CE is a well-known method and can be used in a plethora of analysis areas. It can been use to separate inorganic ions and organic acids, amino acids, peptides and proteins, natural products found in nature such as needles, samples from flowers, leafs, and soil, DNA fragments, single stranded DNA, RNA as well as nucleic acids, drugs, toxins, and chiral compounds (*65*). There are many modes of capillary electrophoresis depending on the desired sample to separate. Capillary zone electrophoresis (CZE) or more often just CE briefly mentioned above is nowadays a well-established analytical method that will be discussed further in this thesis (*vide infra*). In capillary gel electrophoresis (CGE), the capillary is filled with an anticonvective media so solute diffusion is minimized and thereby zone broadening is minimized (*66, 67*). With gels the solute will not adsorb to the capillary wall and electroosmosis (discussed later) is eliminated. It is a good method for separation of proteins. Proteins can also be separated by capillary isoelectric focusing (CIEF) (*68, 69*), where a solution forming a pH gradient is used to separate molecules in a mixture. When voltage is applied, the ions will migrate to a region where they become electrically neutral. After focusing, the zones are mobilized through the capillary. Capillary electrochromatography (CEC), uses a packed column similar to chromatography and can be used to separate peptides (*70*). In addition to separation CE methods can be used for sample preparation. Capillary isotachophoresis (CITP), is an important tool for pre-separation and pre-concentration of trace analytes in complex or diluted samples (*71*). The sample is inserted between two solutions (*72*), one containing a leading and one a terminating electrolyte. The sample concentrates between the electrolyte solutions and the sample bands migrate at the same velocity. Micellar electrokinetic capillary chromatography (MEKC)(*73*), is like CZE; however, a surfactant is added at a concentration above the critical micelle concentration (micelles formation) and the micelles enable separation of neutral molecules by creating a pseudo stationary phase for chromatography. This will also be discussed further later.

3.2 Fundamentals of CE

CE is a fast method that is easy to use, inexpensive, and does not involve complicated equipment. Generally all that is needed are two buffer reservoirs with electrolyte solution and an electrode in each, a fused-silica capillary, often coated in polyimide for support, in contact with the solution of each reservoir for separation, a high voltage power supply connected across the ends of the capillary, and a detector (Figure 6). The fused silica is an open tubular column without any packing material, thus eliminating two of the conditions responsible for zone broadening of peaks in liquid chromatography and so higher separation efficiency is generated. When electricity is passed through a buffer the ions generate heat called Joule heating. Inside a capillary with current flowing through it, heat is generated uniformly in the buffer but the heat is removed at the capillary walls generating a temperature gradient inside the capillary (*74*). This gives rise to zone broadening by changed viscosity and density gradients. Small capillaries have an advantage in enhanced heat dissipation owing to the high surface area to volume ratio. This permits the use of high-potential fields that lead to efficient separations in short time. Thus, with smaller capillary i.d. higher potential fields can be applied and faster separations achieved.

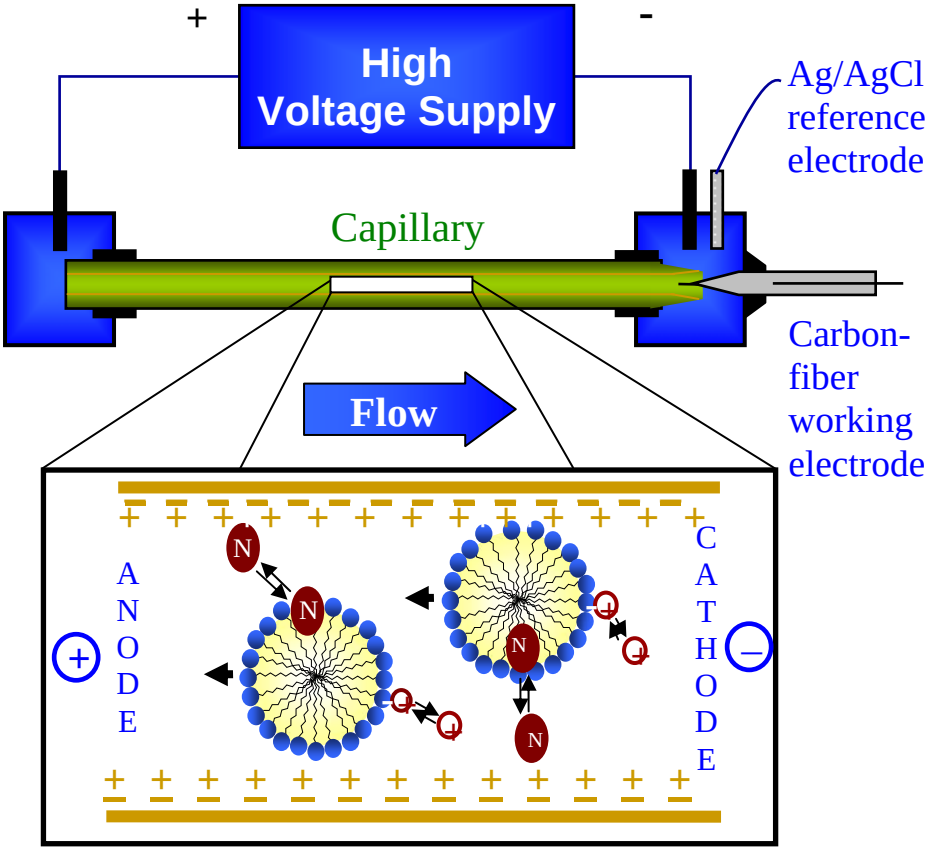


Figure 6. MEKC where ions migrate in an electrolyte solution under the influence of an electric field. Cations are attracted to the negative side (the cathode), anions to the positive side (anode). A surfactant is added at a concentration above the critical micelle concentration (micelles formation) and the micelles enable separation of neutral molecules by creating a pseudo stationary phase for chromatography.

3.3 Electroosmosis and electrophoretic migration

At a pH over ~2 the silanol (Si – OH) groups covering the inside of capillary wall are negatively charged (*75*). The cations in the electrolyte solutions are then attracted to the capillary wall and adsorb to it forming the Stern layer (adsorbed cations and negative charge from the wall). When the potential is turned on, the bulk of the solvent will start to flow towards the cathode (negative potential). The ions in the Stern layer are adsorbed to the walls and will not move. Outside the Stern layer is a diffuse double layer rich in solvated cations, called the slipping plane, which moves with the migration potential. The movement is caused by the small zeta ζ potential formed between the Stern layer and the slipping plane (*76*). The bulk movement of solvent is the electroosmotic flow (EOF) and it will move with a velocity (ν_{EOF}) (Equation 1) when an electric field E (voltage/length of the field) is applied.

$$\nu_{EOF} = \frac{\varepsilon\zeta}{4\pi\eta} E \equiv \mu_{EOF} E \qquad (1)$$

where μ_{EOF} is the electroosmotic mobility, ε is combined for the electric permittivity of vacuum (1 by definition) and the medium, and η is the viscosity. The velocity of the bulk solvent is dependent on the zeta potential and inversely related to the viscosity. The velocity profile of the bulk movement can be seen in Figure 7. The cations in the slipping, layer within ~10 nm from the walls, create the uniform plug-like profile which is the reason for the high separations efficiency by CE (*75*).

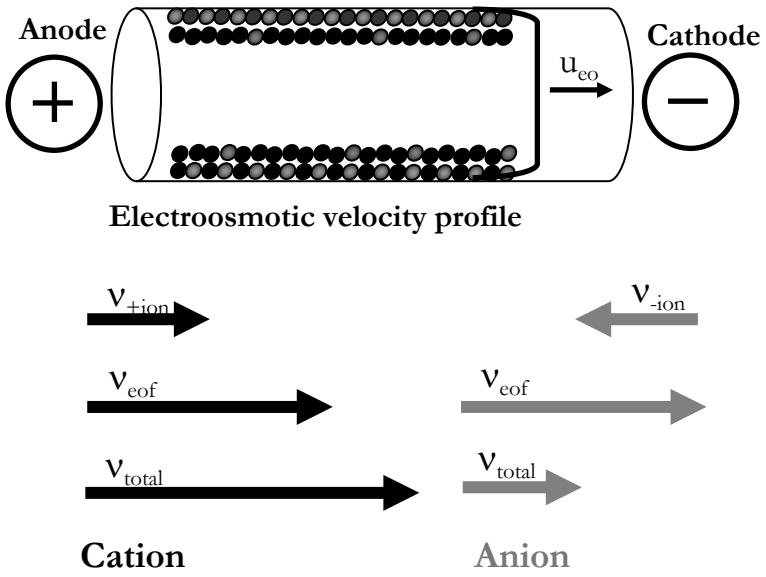


Figure 7. The electroosmotic velocity profile of the bulk movement and total apparent velocity.

The EOF moves the whole bulk, but the ions in the bulk will also be affected by the applied potential. Cations will be attracted to the cathode and the anions to the anode and the ions will move towards the electrodes controlled by their electrophoretic mobility. The velocity of the electrophoretic mobility (EPM), ν_{EPM}, (Equation 2) for a spherical particle of radius r is dependent of the charge of the ion, the strength of the applied field, E, the size of the particle and the viscosity:

$$\nu_{EPM} = \frac{q}{6\pi\eta r} E \equiv \mu_{EPM} E \qquad (2)$$

where μ_{EPM} is the electrophoretic mobility. The ions in the solution will be affected by the sums of two forces, the electroosmotic and the electrophoretic with the total apparent velocity (Equation 3) and mobility (Equation 4):

$$\nu_{total} = \nu_{EOF} + \nu_{EPM} \qquad (3)$$

$$\mu_{app} = \mu_{EOF} + \mu_{EPM} \qquad (4)$$

The cation will electrophoretically move towards the cathode normally in the same direction as EOF creating a velocity greater than the EOF (Figure 7, bottom). Anions are pulled by EOF towards the cathode but their electrophoretic migration is in the opposite direction, towards the anode. The total velocity for an anion is less than the EOF. The differential force for separation of solution species is the EPM while EOF is a force of flow that is the same for all solutes.

The electroosmotic mobility for a neutral species (Equation 5) is the speed $\nu_{neutral}$ divided by the electric field, E:

$$\mu_{EOF} = \frac{\nu_{neutral}}{E} = \frac{L_d / t_{neutral}}{V / L_t} \qquad (5)$$

where L_d is the length of the capillary to the detector, L_t is the total length of the capillary, V is the voltage applied across the capillary ends, and t is the time required for the neutral species to move from the injector to the detector. Since neutral species

lack a charge there is no electrophoretic mobility and neutral species cannot been separated from each other by only zone electrophoresis without modifying the solute. The elution order in CE without any modifications to the rate of EOF is first cations with highest mobility, second, the neural species at the μ_{EOF}, and third anions with the highest mobility will elute last.

3.4 Resolution and theoretical plates

The resolution (R_S) is a measurement of separation efficiency between the peaks of two analytes and can be calculated by Equation 6:

$$R_S = \frac{f \cdot \Delta t}{(w_1 + w_2)} \qquad (6)$$

where f is 1.18 if w is the width at the half height of the peak and 2 if the width of the peak at baseline is used, and Δt is the difference in migration time between the peaks. In **Paper I** where the resolution was calculated w at the width at the half height of the peak was used and an f of 1.18.

A measurement of the efficiency of a separation is the theoretical plate numbers (N). It originates from distillation theory when the high-performing columns had discrete sections called plates in which equilibration was made. The more sections the better distillation. Nowadays in CE there are no true plates, and N is a theoretical value. N can be calculated by Equation 7:

$$N = \frac{L_d^2}{\sigma_{total}^2} = \frac{5.55 t_r^2}{w_{½}^2} \qquad (7)$$

where L_d is the distance to the detector and σ^2 the variance of zone broadening, t_r is the retention time of the peak and $w_{½}$ the width at the half height of the peak. For the use of the width of the peak at baseline the factor is changed to 16.

As mentioned above zone, broadening effects from three of the conditions responsible for zone broadening of peaks in packed columns are eliminated with an open tubular column and without any packing material. These terms are the multiple paths for the analytes around the support material (so-called Eddy diffusion), resistance to mass transfer in the mobile phase (spreading due to laminar flow), and resistance to mass transfer in the stationary phase (which involves the equilibration time of the analytes between the mobile and stationary phases). Terms that still affect

zone broadening in capillaries are longitudinal diffusion, σ^2_{diff}, temperature gradients σ^2_T, sample introduction σ^2_{introd}, and interactions of cations with the silanol groups on the walls of the capillary σ^2_{wall} (*77*). The diffusion is minimal relative to the high migration rates and the temperature is shown not to be a major factor in peak broadening under typical experimental conditions (*78*). According to Huang et al. (*78*) and Jones et al. (*79*) the most significant factor in zone broadening in CE is the sample introduction volume (the plug length). They also conclude a sample plug less than 3 % of the capillary length does not lead to excessive zone broadening. The total effect of the terms on zone broadening (Equation 8) may be summed:

$$\sigma^2_{total} = \sigma^2_{diff} + \sigma^2_T + \sigma^2_{introd} + \sigma^2_{wall} \quad (8)$$

3.5 Injection

For **Papers I-III** electrokinetic injection of the sample was used but another alternative is the hydrodynamic injection where sample is injected by raising the inlet of the capillary above the outlet reservoir. The pressure change drives the solutions in to the capillary, while a potential is applied for the electrokinetic injection. The EOF will pump electrolyte solution and with it the analytes in to the capillary. The injection volume $V_{injection}$ by electrokinetic injection can be calculated by Equation (9):

$$V_{injection} = \frac{E_{injection}}{E_{separation}} \cdot \frac{t_{injection}}{t_{retention}} \cdot V_{capillary} \quad (9)$$

where $E_{injection}$ is the injection potential, $E_{separation}$ is the separation potential, $t_{injection}$ is the time of injection and $t_{retention}$ is the analyte retention time for the analytes. Analytes that move quickly through the capillary will be injected to a greater extent then analytes that moves slowly in the capillary (*76*).

3.6 Buffer composition

The composition of the buffer is the most important factor for many CE separations. Although changing the field strength by changing either the capillary length or the applied voltage can also be effective, changing the buffer composition the separation can be carried out even more effectively. Factors to be considered are the type of buffer, concentration of the ions in the buffer (ionic strength), the identity and concentration of additives such as surfactant, and the pH of the run buffer. With increasing pH the silanol groups on the wall of the capillary are more ionized thus

increasing the EOF. Furthermore the pH can affect the analytes to be separated depending on the dissociation constants.

3.6.1 MEKC

Only ions can be separated with traditional zone electrophoresis. As mentioned earlier an added surfactant to the separation buffer will allow separation of both ions and neutral species as a pseudostationary phase is created in the form of micelles. One surfactant commonly used is the sodium dodecyl sulfate (SDS). It has a negatively charged head-group and a lipophilic (oily) tail of 12 carbons. The SDS molecule aggregates in aqueous buffer solution forming micelles, spheres with the hydrophilic head-group facing outward and the lipophilic tail inward (Figure 6) with a microenviroment of a non-polar solvent. Neutral species interact with these structures more or less, depending of the molecules properties, partitioning in and out of the micelles. Also cations interact with the anionic head-groups of the micelles in an ion exchange manner. The negatively charged SDS micelles migrate in a direction opposite to the electroosmotic flow. This generates a fast moving aqueous phase and a slow-moving micellar phase, or pseudostationary phase. The introduction of the pseudostationary phase adds selectivity in the separation of both neutral and cationic species, but also changes the parameters of zone broadening. Mass transfer in the stationary phase can no longer be ignored, but mass transfer into micelles is fairly fast and the zone broadening effect is modest (*75*).

3.6.2 Borate complexation

Although CE is a powerful separation technique for ions and with the introduction of micelles in the separation buffer it is also effective for separation of neutral species, small ions with similar composition, like neurotransmitters, do not always have large enough differences in their electrophoretic mobility or in their interaction with the micelles to give adequate separations. Borate complexation (Figure 8) with vicinyl hydroxyl groups (cis-diol), a common feature among several neurotransmitters, helps to resolve a larger number of analytes in separations (*80, 81*). The complexation of the catechol group alters the charge by adding a net negative charge and the increased size of the borate–analyte complex changes the partitioning behavior with the micelles. Complexation of neurotransmitters with vicinyl hydroxyl groups results in a change in the charge-to-mass ratio substantial enough to allow the separation of analytes which are poorly separated under conditions without borate.

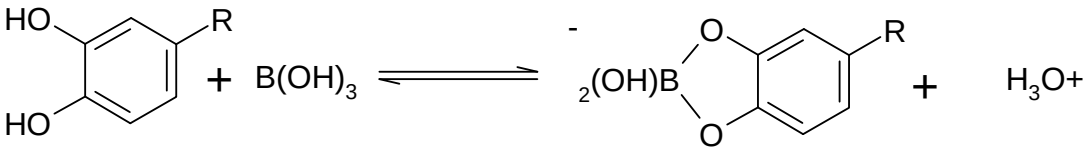


Figure 8. Borax complexation with vicinyl hydroxyl groups on a catechol-group.

3.6.3 Henderson-Hasselbalch

In **Paper I** the pH was optimized in the separation buffer by carefully controlling the addition of sodium hydroxide. Small aliquots of sodium hydroxide were added to the separation buffer, made with the weak acid borate, prior to separation and the resolution of the peaks gradually improved. After acquiring optimum peak resolution, the pH of the buffer was calculated by the Henderson-Hasselbalch Equation (10) where K_a is the acid dissociation constant of 9.14 for borate acid.

$$pH = pK_a + \log\left(\frac{[A^-]}{[HA]}\right) = 9.14 + \log\left(\frac{[H_2BO_3^-]}{[H_3BO_3]}\right) \qquad (10)$$

3.7 *In vivo* sample preparation

Cocaine is an addictive and harmful psychoactive drug that has been rated the second most addictive and the second most harmful drug by psychiatrists specialized in the area of addiction (*82*). The blockage of the dopamine transporter and the increased extracellular dopamine concentration is in part the reason for the experience of wellbeing and euphoria that is characteristic with this drug. Despite extensive knowledge of how cocaine affects the body an effective drug treatment for cocaine addiction has yet to be found. Methylphenidate has been used in the treatment of attention deficit hyperactivity disorder (ADHD) for more than 60 years (*83*). It binds to the dopamine transporter where dopamine is transported into neurons and increases extracellular dopamine (Figure 2) levels. This mechanism is similar to that proposed for cocaine (*84, 85*), but methylphenidate is thought to elicit fewer addictive and reinforcing effects owing to its slower pharmacokinetics. It has been proposed and tested (*86*) that substitution of methylphenidate for cocaine can be used as an approach in cocaine treatment, but more knowledge about the mechanism of action is needed. In **Paper IV** *in vivo* voltammetry experiments in *Drosophila* were conducted to explore this mechanism of the two drugs cocaine and methylphenidate on the transporter function.

In the experiments performed, the fly was positioned in a fly collar, a 38.1 mm diameter concave plexiglass disk with a 1.0 mm hole in the center. Low melting point agarose was applied to hold the fly in place. Preparation of the fly for the experiment was as follows: a cut was made along the line of one eye, next just over or between the three dorsal ocelli (light-sensitive organs), and a third cut along the second eye. These three cuts generate a small lid and by lifting the lid a forth cut was made to remove the cuticle part and expose the brain. Before exposing the brain to the environment, a buffer containing collagenase was added to protect the brain from the air as well as relax the extracellular matrix in the brain. After 15 min of collagenase treatment the solution on top of the open fly brain was changed to a buffer solution mimicking the extracellular matrix. A carbon fiber microelectrode, 50 μM in length, was inserted in the mushroom body and a microinjector containing dopamine was positioned just outside. Dopamine was applied and diffused through the brain and was detected by the electrode, see below. (Figure 9)

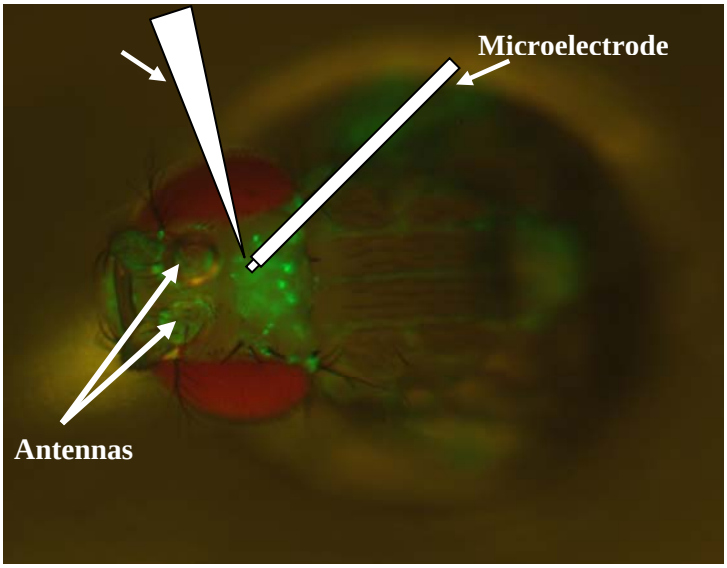


Figure 9. Top view of *Drosophila* head mounted in a collar. Microelectrode and injection pipette (structure marked with arrow in the middle) are painted to show position. Dopamine neurons are visualized in green with GFP

3.8 Detection

Electrochemical detection with carbon fiber microelectrodes (*87*) offers high spatial and temporal resolution, are inexpensive, and is relatively simple to carry out (*88*). Ralph Adams and his group were the first to measure *in vivo* concentrations of neurotransmitters with a carbon microelectrode and electrochemical methods (*89, 90*). With that he started the successful field of *in vivo* electrochemistry in the brain. They soon noticed that not only neurotransmitters are oxidized at a carbon

microelectrode in the brain and they realized the importance of identifying and confirming the chemical source of the oxidation currents observed (*91*). Separation of brain samples with CE and detection with carbon fiber microelectrodes together with standards for comparison have been used to identify and confirm the chemical sources for the current work.

3.8.1 Amperometry

Detection in **Papers I-III** was carried out electrochemically with end-column amperometric detection. This was preformed by inserting a carbon fiber microelectrode into an etched end of the capillary (*92*). The etched end has two advantages; first it ensures proper alignment of the electrode as well as it helps to maintain the alignment during the separation. Second, the electrode must be isolated from the high potential field used for electrophoresis and the etched end provides this. The carbon fiber electrode is made of a carbon fiber with a diameter of 5 µm (Figure 10 A). The electrode is cut to a total exposed length of 500 µm. The rest of the carbon fiber is sheltered by a glass capillary and filled with conductive solution. At the top of the carbon fiber a metal wire is inserted to make electrical connection with the carbon fiber. When electroactive neurotransmitters and metabolites exit the separation capillary they hit the electrode. The electrode is held at a constant potential of +0.70 V versus a reference electrode (Ag/AgCl). The substances detected will at this potential oxidize generating a current flow and result in a peak (Figure 10B).

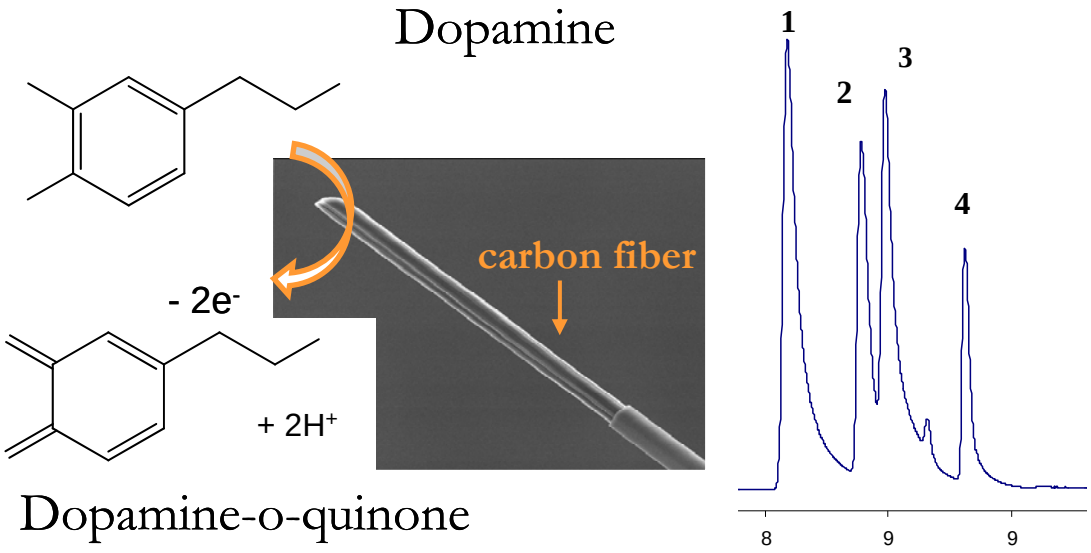


Figure 10. A) Oxidation of dopamine over a carbon fiber microelectrode. **B)** Peaks representing dopamine (1), salsolinol (2) , norsalsolinol (3) and N-acetyloctopamine (4).

Using the area under the peak, the detected neurochemicals can be quantified (*92*) by use of Faradays equation:

$$N = Q/nF \qquad (11)$$

where N is the number of moles of compound oxidized, n is the number of electrons transferred in the oxidation reaction, and F is the Faraday constant. An advantage with electrochemical detection is that only electroactive species are detected by the electrode which means co-elution of non-electroactive species will not disturb detection. Substances in the fly homogenates detected electrochemically have also been successively separated with the also commonly used method for sample separation, high-performance liquid chromatography (HPLC) (*93-98*).

3.8.2 Voltammetry

In **Paper IV** fast scan cyclic voltammetry (FSCV) was used for the experiments performed. Since some neurotransmitters have specific oxidation/reduction potential, FSCV can be used to identify species. This has been used when coupled to CE for detection of neurotransmitters in *Drosophila* larva (*61*). Instead of keeping the potential fixed as is done with amperometric detection in FSCV the potential is scanned back and forth in a cycle. This scanning of the cycle is carried out at high speed (200 V/s) and repeated constantly at 10 Hz. The scanning of dopamine starts at -0.4 V and is positive to 1.0 V and back again. During the forward scan dopamine is oxidized to dopamine-orthoquinone and the quinone is reduced back to dopamine during the backward scan. This generates both an oxidation and reduction current as well as it clears out the toxic dopamine-orthoquinone product. An electrode in an electrolyte at each potential has a characteristic surface charge. This charge will increase with increasing scan rate, generating a high background current (*88*). This background can be removed by background subtraction and this has been used in **Paper IV**. After recording many voltammograms over time a false-color plot (Figure 11) is made where the x-axis is time and the y-axis is the potential scan. The color is the z-axis representing the current. A current vs time plot can be achieved by reading the plot horizontally at a specific potential and voltammograms are read from the plot by reading it vertically as current vs voltage.

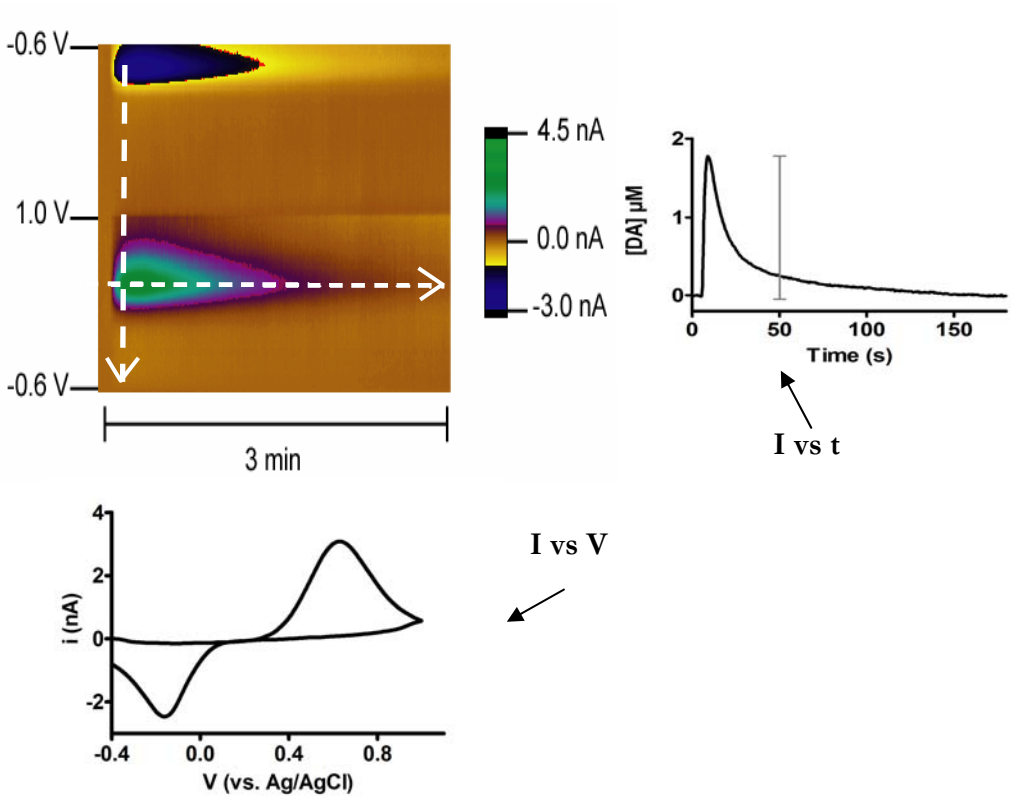


Figure 11. Color plot: a current vs time plot can be achieved by reading the plot horizontally at a specific potential and voltammograms are read from the plot by reading it vertically as current vs voltage.

In this work *in vivo* voltammetry in the adult fly was used; however, Jill Venton's group has also carried out detection of dopamine *in vivo* in *Drosophila* larvae with FSCV (*99*). For those experiments the electrode was inserted in the neuropil area of the vNC (Figure 5A). They have also detected serotonin (*100, 101*) with the same method in the larva of *Drosophila. In vivo* voltammetry is an effective method to detect and monitor neurotransmitter contents in the brain of *Drosophila* melanogaster

4 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

This thesis covers an area of analytical chemistry which can be used in many diverse areas of life science even though the focus here has been towards the neuroanalytical field. The fly is a fantastic model as it is so simple but provides useful information applicable to systems as complex as humans!

In previous work, the flies were exposed to alcohol fumes to evaluate the time needed to intoxication after repeated exposures. Tolerance to the alcohol was developed and reached a maximum after approximately 10 days. With the advances presented here including the improved resolution in separation from changing the run buffer and the improved sample preparation methods, the effects of alcohol tolerance on biogenic amine levels might now be quantified. We have already shown in **Paper I** that six neurotransmitters thought to be involved in response to alcohol can be separated and quantified. It is now relatively simple to set up the fly inebriometer again and expose the flies to alcohol with daily regiments. There are also alcohol mutants from the Heberlein group that could prove very useful. These mutants, the mutant *barfly* with reduced sensitivity to alcohol, and the *tipsy* with increased sensitivity to alcohol, could be used to study the mechanisms that regulate acute sensitivity to ethanol (*102*). Also they have studied the mutant *Hangover*, with reduced development of ethanol tolerance in *Drosophila* and found that it engages two systems that function in parallel; one involves octopamine (*103*) which is electroactive. Furthermore these fly mutants might be used to examine the clearance of exogenously applied dopamine as measured in the *in vivo* experiments since ethanol is thought to stimulate dopamine release in rats (*104*).

And why stop with alcohol? There are numerous of drugs the fly could be exposed to that are of interest in addiction and drug abuse. Cocaine and methylphenidate have been used for the *in vivo* experiments in **Paper IV**, just to mention two. Some preliminary experiments carried out with CE coupled to mass spectrometry show that methylphenidate fed to flies might affect more than just dopamine concentration and the question is will a cocaine treated fly also show a change in neurotransmitter related metabolites that are not just in the dopamine system?

There are many interesting mutants showing various aspects of the disease progression for the neurodegenerative diseases including Alzheimer's disease,

Huntington's disease, and Parkinson's disease. It would be interesting to see how the neurotransmitter content changes in comparison to wild-type flies in those models. Also CE coupled to a mass spectrometry could be used to detect changes in the levels of neurotransmitters involved along with the co-eluted proteins and lipids which cannot be detected electrochemically.

It is possible to sample the extracellular fluid with CE and to detect species with electrochemical detection (*52*). It would be interesting to combine this with the *in vivo* experiments. Shippy and his group have shown sampling of hemolymph fluid (*62, 63*) from both fly larvae and adults, so it would be interesting to sample extracellular fluid from the mushroom body. The methods to this exist and just need to be combined.

The *in vivo* method presented in this thesis also should be of use in the future. I showed that the effect of cocaine on the dopamine transporter was almost non-existent after oral methylphenidate. It would be interesting to expand this work to more neurotransmitters in the fly brain than dopamine. These other transmitters would need to be those that can be detected with electrochemical detection and this would require use of more anatomical areas of the brain than the mushroom body. Also there is a fly mutant used by Jill Venton's group (*105*), and in the possession of our group in which the mutant containing dopaminergic neurons can be stimulated with (TH-GAL4/UAS: ChR2 genotype). This seems to work well in studies shown by the Venton group for the larval experiments and it would be open many possibilities for the study of stimulated release in the adult if it can be effectively used.

5 SUMMARY OF PAPERS

There were two aims with this thesis. The first was to improve the separation and quantification of neurotransmitters in the adult *Drosophila melanogaster* with capillary electrophoresis. An optimization of previous protocols would make the technique useful in the investigation of effects of drugs of abuse on neurotransmitter levels with the fly as the model and these optimizations are explained in **Papers I-III**. The second aim was to go a bit deeper in the understanding of dopamine system and how the psychoactive drug cocaine and the ADHD drug methylphenidate affect the dopamine transporter. This work was carried out by use of an *in vivo* method with fast scan cyclic voltammetry and is explained in **Paper IV**.

In P**aper I** the protocol previously used to separate fly head homogenates was optimized. A new method to control the pH in the buffer was presented and an optimum pH for separation was attained. Since most of the neurotransmitters of interest and their metabolites have a pK_a between 9 and 10, a small change in the running buffer has a large impact on the separation selectivity and resolution of some of the analytes of interest. This required pH changes were so small that they were elusive to detect with a pH meter. Hence, a method was developed where a fixed amount of base was added to running solutions until the desired separation resolution was achieved and the pH was back calculated. In the previous work 14 neurotransmitters and their metabolites and precursors were separated in a standard solution. With the new protocol and running buffer, we were able to separate 23 neurotransmitters and their metabolites and precursors. In the fly homogenate the focus was on six of the substances thought to be involved in response to alcohol. Dopamine, salsolinol, norsalsolinol, N-acetyloctopamine, octopamine, and N-acetyldopamine were identified. The detection of salsolinol and norsalsolinol in the fly is thought to be novel.

Examining whole head homogenates allows analysis of a high number of flies per homogenate sample and also helps to reduce the fly-to-fly variability. Unfortunately, it provides a significant matrix effect, which interferes with neurotransmitter quantification. In **Paper II**, we showed that this matrix effect mainly originates from the eye pigments, as they are naturally highly electroactive. Mutant flies lacking the red eye pigment were tested as an alterative to the red eyed Canton-S. Quantification of the neurotransmitters from dissected brains instead of whole heads showed that

the neurotransmitter content in the white type flies was different from that in the Canton-S flies indicating head homogenates from the white type flies could not be used for control experiments. The quantification of the peaks was improved considerably by dissecting the brains and thus avoiding the effects from substances in the eyes and cuticles. In addition, the levels of dopamine and octopamine in different regions of the brain were measured showing the highest dopamine concentration in the PAM region and octopamine could be measured in both the central lobe and in the optic lobe at equal concentration but was not observed in the PAM region.

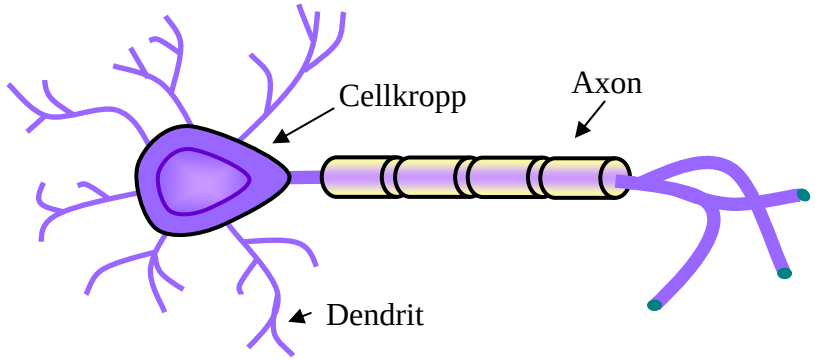
The brain of a *Drosophila melanogaster* is tiny (< 1 mm^3) making the process of hand dissection of the even smaller brain time consuming, and dissection does not always result in success as the small structures easily break. Even with a successful dissection it is hard to obtain a sample with a large number of brains as the brain samples begin to degrade as soon as they are removed from the head. *Drosophila* mutants have been developed to study neurodegenerative diseases such as Alzheimer's disease (*106-109*), Huntington's disease (*110*), and Parkinson's disease (*111*). Many of the neurotransmitters associated with these diseases occur in minute amounts that can be difficult to detect even before degradation. As such, it is essential to develop methods that provide quick and accurate analyses on these unique biological systems. A method of freeze drying the brains of *Drosophila* was accomplished for increasing the sample size and this is described in **Paper III**.

In **Paper IV** a completely different approach was used to examine the effects of drugs in *Drosophila. In vivo* experiments were used to explore dopamine dynamics and how two drugs affect its transporter function. More specifically I investigated how the dopamine transporter in the adult *Drosophila* is inhibited by cocaine and the ADHD drug methylphenidate (Ritalin). Cocaine blocks the dopamine transporter increasing the extracellular dopamine, and this is in part the reason for the experience of wellbeing and euphoria that is characteristic with this drug. Cocaine is also highly addictive. It has been proposed that methylphenidate could be used in the treatment of cocaine addiction as it might competitively block dopamine uptake and has slower pharmacokinetics. The data in **Paper IV** show that treatment with oral methylphenidate in a concentration dependent manner blocks the effect of cocaine on the dopamine transporter in *Drosophila* melanogaster and suggest this system as a model to study this interaction.

6 POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Statisk och dynamisk mätning av neurotransmittorer från *Drosophila* hjärna.
Den lilla bananflugan som surrar runt frukten som blivit bortglömd på bänken i köket är så mycket mer än en irritationsfaktor. Bananflugan, kallad *Drosophila melanogaster* på latin, har sedan tidigt 1900-tal används som en modell för att förstå genetik och på senare tid underlättat förståelsen runt sjukdomsförloppen i neurodegenerativa sjukdomar såsom Alzheimers, Huntingtons och Parkinsons sjukdom.

Nervceller, eller neuroner kommunicerar med sig själva och sin omgivning via små signalsubstanser, så kallade neurotransmittorer. Utan dessa signalsubstanser fungerar inte vårt känsloliv, tankeverksamhet, vår möjlighet till motorik, eller grundläggande funktioner som reglering av sömn. Det räcker med en liten balansrubbning av neurotransmittorhalten i hjärnan för att ge stora effeker på individen som tex de nyss nämnda neurodegenerativa sjukdomar eller ge upphov till depression eller missbruk. Nervcellens uppgift är att ta emot, leda vidare och sända ut nya signaler och det kan den göra i och med dess fysiska utseende. En nervcell består av tre delar, en cellkropp, ett antenn-nätverk bestående av dendriter vilka tar emot signaler och ett axon med förgreningar i änden för att sända ut signaler. Cellerna kommunicerar med varandra genom att axonet från en cell sänder ut neurotransmittorerna som dendriterna känner av och skickar vidare till nästa cell. När vi lär oss något nytt ändrar nervceller sina nervbanor och bildar nya kopplingar. Eftersom man inte vill eller kan utföra experiment på människor använder man sig av modeller. Bananflugan är en av dem. Det kan låta konstigt att man kan använda den information som man fått tillhanda genom experiment med flugor då storleken på dess nervsystem är så många gånger mindre än däggdjurs, men faktum är att flugan har visat sig kunna uppvisa avancerade hjärnfunktioner såsom att lära sig från triggade instinkten att undvika och äta och komma ihåg information de lärt sig från tidigare erfarenheter .



Figur 1. En nervcell med dess tre delar, dendrite, cellkropp och axon.

Här i denna tes har jag velat förbättra analysen av neurotransmittorer från flughjärna för att kunna detektera så mycket och så många av neurotransmittorerna som möjligt. Proverna har separerats med kapillärelektrofores, en metod som klarar av små provvolymer som de från bananflugehjärnan och dessutom separerar dem på kort tid med bra separationsförmåga utan att kräva för komplicerad eller dyr utrustning. För detektion har elektrokemi använts vilket är möjligt eftersom neurotransmittorer är elektroaktiva. Att de är elektroaktiva betyder att man kan utsätta neurotransmittorerna för en spänning och som respons avger neurotransmittorerna ett flöde av elektroner (ström) som kan detekteras av en elektrod.

I den första artikeln, **Artikel I**, förbättrades separationen genom att ändra pH på den buffertlösning som transporterar ämnena genom kapillären och på så sätt kunde 23 neurotransmittorer separeras och identifieras. Vi använde oss av denna buffert för att titta närmare på sex neurotransmittorer som anses vara involverade vid alkoholintag. Två av dessa har man mig veterligen inte kunnat se tidigare med vår detektionsmetod. För dessa experiment användes hela flughuvuden som krossades innan separation. Problem som uppstår med att använda hela huvudet är att pigmenten från de röda ögonen också är elektroaktiva och stör analysen av neurotransmittorerna som finns i små mängder jämfört med pigmenten från ögonen.

För att försöka komma runt detta gjordes experiment i **Artikel II** med en mutant som saknar ögonpigment (vita ögon). Tyvärr visade det sig att denna mutant hade avikande koncentrationer av neurotransmittorer från standardflugan och kunde därför inte användas. Däremot kunnde en mutant för visualisering av neurotransmittorn dopamins nervceller användas då denna typ hade neurotransmittorhalter som mer överensstämde med standardflugan. Det finns, i alla flugtyper, en liten mängd av neurotransmittorn dopamin i det tjocka hudlagret (skinnet) så hela huvudet är inte optimalt att använda om man vill studera just dopamin. En metod för att dissekera ut hela hjärnan användes i stället och halterna av neurotransmittorer analyserades i både hela hjärnan och i olika regioner av hjärnan. Det visade sig att halten dopamin är störst i den region där dopaminneuroner är lokaliserade.

Det är tidsödande att dissekera ut hjärnorna för hand och tiden är viktig då hjärnorna snabbt börjar brytas ner så fort de tas ut och utsätts för luft. För att kunna få en större mängd hjärnor på kortare tid användes en metod i **Artikel III** för att frystorka hjärnorna. Frystorkning leder till att de processer som bryter ner hjärnan stoppas upp såväl som att det är lättare att dissekera ut hjärnorna och större provmängd uppnås.

Dessutom kunde proven koncentreras bättre och på så sätt ge tydligare utslag vid analysen.

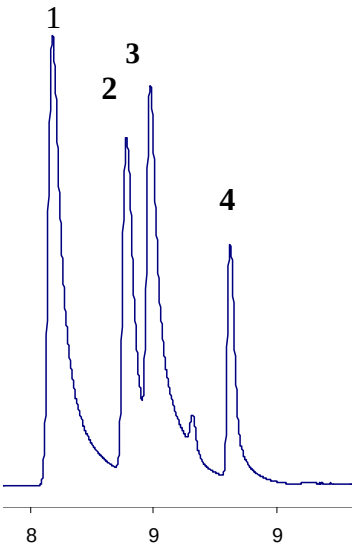


Figure 2. Exempel på hur ett uslag vid analysen kan se ut där fyra neurotransmittorer analyseras.

I den sista artikeln, **Artikel IV** studerades mekanismen för hur dopamin transporteras tillbaka till nervcellen igen, via den så kallade dopamintransportören. Dopaminetransportören blockeras av kokain så att dopamin inte kan transporteras in i cellen utan ackumeleras utanför cellerna istället. Detta är orsaken till det tillfälliga rus och välbefinnande som kan upplevas vid kokainintag. Kokainmissbruk är ett stort problem i hela världen och det är svårbehandlat. Det har föreslagits att ADHD-medicinen Ritalin, kemiskt kallad metylfenidat, kan användas i kokainmissbruksbehandling då den blockerar dopamintransportören. Det visade sig att bananflugans dopamintransportör blir blockerad av metylfenidat och att blockeringen är koncentrationsberoende. För den högsta koncentrationen av metylfenidat som användes i flugan var kokainets effekt nästintill obefintlig vilket tyder på att metylfenidat blockerar kokains effekt i bananflugan.

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"No man is an Island, entire of itself; every man is a piece of the Continent, a part of the main…" These famous words where written by the English clergyman & poet John Donne in Meditation XVII from 1624 and I think they are appropriate here. When you do science you are a piece of the research "continent". During the time I did my PhD I interacted with so many persons, scientist as well as non-scientist, I was not an "Island" and I would like to thank them.

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Live Long and Prosper!
Carina

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