# Thesis for Degree of Doctor of Philosophy

# The Mechanisms of Exocytosis Studied in Cells and Models with Amperometry

## Lisa Mellander

UNIVERSITY OF GOTHENBURG

Department of Chemistry and Molecular Biology
University of Gothenburg
Gothenburg, Sweden
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LISA MELLANDER

Department of Chemistry and Molecular Biology
University of Gothenburg
SE-412 96 Göteborg
Sweden

Cover picture: Schematic of amperometric detection of exocytosis at a neuroendocrine cell.

*To Mike and Julia*

# ABSTRACT

Exocytosis is the universal basis for neuronal communication, allowing the controlled release of neurotransmitter molecules from the presynaptic cell. Exocytosis has been widely studied in many systems. However, the details of this vital process are not fully understood. Exocytosis is quantal in its nature in that the neurotransmitter is released in packages. These packages were initially assumed to be comprised of the entire vesicular content. More recently, this simplistic view has been replaced with a more complex one with several different suggested modes of release. Vesicles can, in addition to the irreversible complete collapse of the vesicle into the cell membrane, fuse transiently with the cell membrane in a process that has been termed kiss-and-run. Transient fusion has the advantage of allowing the vesicle not to release its entire content but instead potentially control the fraction of neurotransmitter molecules it lets out. To elucidate the details of the exocytotic process, it is necessary to resolve individual release events. Amperometric detection at micrometer sized electrodes is an excellent tool for this purpose, providing quantitative and detailed kinetic information about single release events. In this thesis I have employed amperometry to study the mechanisms of exocytosis at PC12 cells and in an artificial cell model.

In paper I, detection of exocytosis using amperometry and fast scan cyclic voltammetry at a microelectrode array is compared and evaluated. In paper II, amperometric detection of exocytosis is compared at a disk and a ring-shaped microelectrode, and the results are used to evaluate the diffusion coefficient of dopamine in the extracellular matrix surrounding the cell. In paper III, I define and explore a new feature of amperometric peaks recorded at PC12 cells, the post spike foot. This feature is used to show that changing the lipid composition of the cell membrane can alter the fraction of neurotransmitter released per event. In paper IV the influence of membrane lipid composition on lipid nanotube dimensions is studied using amperometry at a lipid nanotube-liposome network. It has been demonstrated that sorting of membrane lipids based on structure occurs and a dynamic method for controlling lipid nanotube diameter has been established. The same experimental setup is used in paper V, but here it is applied to PC12 cell plasma membrane vesicles and used to study the membrane dynamics of exocytosis. In this system it has been demonstrated that release occurs though two distinct mechanisms; full and partial distension of the initial fusion pore.

# POPULÄRVETENSKAPLIG SAMMANFATTNING

Nervsystemet är uppbyggt av kedjor av nervceller vilka styr våra medvetna och omedvetna kroppsliga funktioner. Då vårt nervsystem ska skicka en signal krävs det att denna passerar mellan de olika cellerna i signalkedjan. Detta sker främst genom frisättningen av transmittorsubstanser vilka är paketerade i membranomslutna bubblor, så kallade vesiklar, inuti cellen. Frisättningen sker genom att vesikelns membran smälter samman med cellens plasmamembran i en process som kallas exocytos. Detta resulterar i en lipidomsluten kanal mellan vesikeln och cellens utsida. Genom denna kanal kan transmittormolekylerna ta sig ut ur cellen där de kan binda till receptorer på andra celler och på så sätt påverka dessa att skicka signalen vidare. Det är inte känt ifall vesikelns membran helt kollapsar in i cellens membran eller om den behåller sin form och kanalen stängs igen efter utsläppet av molekyler. Om vesikeln stängs igen innebär det en möjlighet för cellen att reglera hur mycket av vesikelns innehåll som frisätts. Detta kan vara en viktig väg för cellen att justera intensiteten av signalen den skickar ut. För att studera detta fenomen krävs det extraordinära analytiska tekniker. Detta eftersom mängden transmittorsubstans som släpps ut är liten och eftersom det sker väldigt snabbt. I den här avhandlingen har jag använt mig av en elektrokemisk metod som kallas amperometri för att studera olika aspekter av exocytosprocessen. Genom att placera en kolfiberelektrod intill en cell som frisätter signalsubstans kan vi detektera de molekyler som släpps ut från cellen. Detta sker genom att elektroden hålls vid en potential där molekylen av intresse lämnar ifrån sig elektroner. Ett enskilt frisättningsevent detekteras då som en strömtopp som orsakas av det ökade flödet av elektroner. Denna topp ger oss bland annat information om hur många molekyler som släppts ut och under hur lång tid frisättningen skedde. Genom att studera levande celler har jag kunnat visa att frisättningen från våra celler sker främst genom en tillfälligt formad por vilken återförsluts efter att en del av vesikelns innehåll har släppts ut. Jag kunde även demonstrera att andelen substans som frisätts kan påverkas genom att ändra cellens membransammansättning. Detta kan vara en möjlig väg för cellen att reglera de signaler den skickar ut och därmed medverka till cellens "minne".

# PART A: TABLE OF CONTENTS

# PART B: LIST OF PUBLICATIONS

This thesis is based on the following papers listed, which are referred to in the text by their Roman numbers.

Paper I   B. Zhang, M. L. A. V. Heien, M. F. Santillo, **L. Mellander**, A. G. Ewing, Temporal resolution in electrochemical imaging on single PC12 cells using amperometry and voltammetry at microelectrode arrays. Anal. Chem. 83, 571-577 (2011)

Paper II   R. Trouillon, Y. Lin, **L. J. Mellander**, J. Keighron, A. G. Ewing, Evaluating the diffusion coefficient of dopamine at the cell surface during amperometric detection: disk vs. ring microelectrodes. Provisionally accepted at Anal. Chem.

Paper III   **L. J. Mellander**, R. Trouillon, M. Svensson, A. G. Ewing, Amperometric post spike feet reveal most exocytosis is via extended kiss-and-run fusion. Nature Scientific Reports 2, 907 (2012)

Paper IV   M. E. Kurczy, **L. J. Mellander**, N. Najafinobar, A-S. Cans, Composition based strategies for controlling radii in lipid nanotubes. Submitted to PLoS ONE.

Paper V   **L. J. Mellander**, M. E. Kurczy, N. Najafinobar, J. Dunevall, A. G. Ewing, A-S. Cans, Observation of two modes of exocytosis in an artificial cell model. Manuscript in preparation.


# RELATED PAPERS NOT INCLUDED IN THIS THESIS

**L. Mellander**, A. S. Cans, A. G. Ewing, Electrochemical probes for detection and analysis of exocytosis and vesicles. Chemphyschem, 2010, 11, 2756-2763

K. L. Adams, M. M. Maxon, **L. Mellander**, R. H. S. Westerink, A. G. Ewing, Estradiol inhibits depolarization-evoked exocytosis in PC12 cells via N-type voltage-gated calcium channels. Cell. Mol. Neurobiol., 2010, 30, 1235-1242

# CONTRIBUTION REPORT

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

Paper I    Analyzed some of the data and took part in writing the manuscript.

Paper II    Planned and performed experiments with disk electrodes for original data set, analyzed some of the data and participated in interpretation of the data and writing of the manuscript.

Paper III    Planned and performed the experiments, analyzed and interpreted the main data, and wrote the first draft of the manuscript as well as editing in collaboration with others.

Paper IV    Planned and performed part of the experiments, was involved in analyzing and interpreting the data in collaboration with MEK and participated in outlining and writing the manuscript in collaboration with MEK.

Paper V    Planned and performed the experiments together with NN and MEK, led the team to analyze and interpret the data, and wrote the first draft of the manuscript in collaboration with MEK.

# LIST OF ABBREVIATIONS

Abbreviations commonly used in this thesis:

AA – Arachidonic acid
ACh – Acetyl choline
ADHD – Attention deficit hyperactivity disorder
CSN – Central nervous system
DAT – Dopamine transporter
FSCV – Fast scan cyclic voltammetry
GUV – Giant unilamellar vesicle
LDCV – Large dense core vesicle
LPC - Lysophosphatidylcholine
PC - Phosphatidylcholine
PC12 – Pheochromocytoma cell line
PE - Phosphatidylethanolamine
PNS – Peripheral nervous system
SNARE – Soluble NSF attachment protein
SSV – Small synaptic vesicle
SUV – Small unilamellar vesicle
VMAT – Vesicular monoamine transporter

# 1. INTRODUCTION

## 1.1. The Nervous System

The nervous system can be divided into two major parts, the central nervous system (CNS) and the peripheral nervous system (PNS), where the brain and the spinal cord make up the CNS and the PNS is comprised of the autonomic and somatic nervous systems. The autonomic nervous system controls our inner organs and glands while the somatic nervous system passes signals between the CNS and the skeletal muscles, allowing us to control our movements. The brain communicates with the nerves of the PNS through the spinal cord, which consists of 31 pairs of protruding nerves. The brain consists of a number of different cell types that can be divided into neurons and glial cells. Glial cells are further divided into astrocytes, oligodendrocytes and microglia. The functions of glial cells are vital and involve, but are not limited to, maintaining the structure of neurons and insulating them from one another, cleaning up the extracellular space and storing and providing energy to neurons. However, for the scope of this thesis, I will focus on the nerve cell.

## 1.2. Neurons

The cells that are responsible for transporting the neuronal signals through the nervous system are called neurons. Characteristically, one axon and many dendrites extend from the cell body, which is termed the soma.[1] There are however exceptions, for example there are nerve cells where the axon is the only protrusion, or cells with one axon and one dendrite. The dendrites mainly function as antennas, receiving signals from other cells, whereas the axon passes the received signal on to the next cell. The complexity of the nerve cells and the connections they make with each other was visualized in the late 19th century by Santiago Ramón y Cajal who provided early evidence for the neuronal doctrine, which states that the nervous system is made up of individual cells (Figure 1).
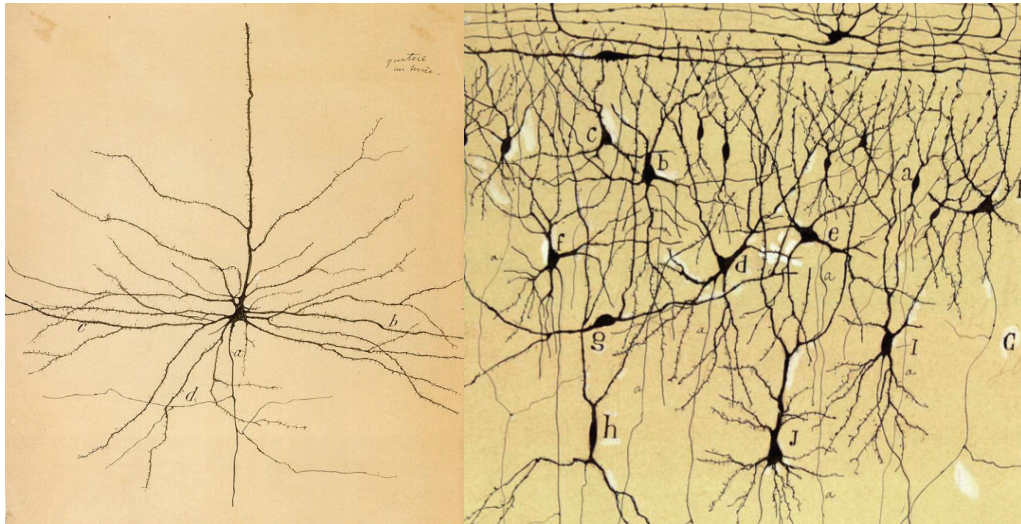
*Figure 1: Neurons as imaged by Santiago Ramón y Cajal.[2]*

One feature present in the nerve cell (actually in all types of cells) is an electric potential of about 70 mV across the cell membrane, with the inside of the cell negative with respect to the outside.[3] This potential is caused by an uneven distribution of ions, with high levels of sodium outside the cell and high levels of potassium on the inside. The distribution of these ions is established by the $Na^+/K^+$-ATPase that pumps sodium out of the cell and potassium into the cytoplasm. Additionally, the membrane is more permeable to potassium than to sodium, ensuring that sodium concentrations remain low inside the cell while some potassium escapes the cytoplasm, resulting in a net negative charge on the inside of the plasma membrane compared to the outside. The resulting potential is crucial for the nerve cell since it allows the transfer of an electrical signal along the membrane, in the form of a sequential depolarization. This progression of a depolarization is called an action potential and it is initiated at the beginning of the axon at a structure known as the axon hillock. The progression of the signal along the axon is made more efficient by the fact that the axon of the neuron often is wrapped in a sheet of myelin. This sheet consists of oligodendrocyte cells that wrap around the axon forming several layers of insulation composed of plasma membrane. These myelin sheets give the axons a white appearance, which allows the CNS to be divided into grey and white matter, where the grey matter is the cell bodies and the white matter is the axons. The insulation is necessary to obtain a fast enough transport of the signal flowing along the membrane of the axon. However, as the action potential reaches a synapse, a different approach is necessary for transferring the signal to the next cell.

## 1.3. Neuronal communication

The brain is comprised of around 100 billion nerve cells and the average cell communicates directly with 1000 other cells.[4] The traditional view of communication between neurons, and the most predominant one, is that it takes place in a structure called the synapse, which is a close contact between two nerve cells.[1] There are synapses where the two connecting cells communicate through direct contact via so called gap junctions, which are pores formed in the membrane between cells, allowing for the exchange of material. These structures are called electrical synapses since it allows for the direct transfer of the electrical signal from the pre- to the postsynaptic cell. However, the most common synaptic transmission occurs through the release of neurotransmitter molecules from the presynaptic neuron into the synaptic cleft. These molecules diffuse across the synaptic gap and bind to receptors on the postsynaptic neuron. This type of signaling is called wiring transmission. A signal between nerve cells can be either excitatory or inhibitory depending on the type of receptor that is present on the postsynaptic membrane. In the case of an excitatory signal the receptor depolarizes the membrane potential. This can occur through the opening of ligand gated ion channels or via the induction of an intracellular cascade leading to the subsequent opening of ion channels depolarizing the membrane. An inhibitory signal typically leads to the hyperpolarization of the postsynaptic membrane. This can occur via the opening of chloride channels leading to an influx of chloride ions or the opening of potassium channels allowing a higher flux of potassium ions out of the cell also resulting in an increased potential over the membrane. The hyperpolarization makes it more difficult for excitatory signaling molecules to depolarize the membrane therefore inhibiting the signaling pathway.

A second mode of neuronal communication through the release of neurotransmitter involves the release at a site outside the synapse, or release in the synapse and subsequent spill-over, resulting in the diffusion of the released molecules over a large area. This type of signaling is called volume transmission, and is a relevant mode of communication for many neurotransmitter molecules. The major effect of volume transmission is neuromodulation.[5] There are many pathways for presynaptic neuromodulation, including effects on the firing rate of the presynaptic cell and alteration of the amount of neurotransmitter inside the vesicles, while postsynaptic effects include altered affinity of receptors and alteration of ion channel conductance.

## 1.4. Neurotransmitters

There is a multitude of neurotransmitters and more are being unveiled still today. The first neurotransmitter to be discovered was acetylcholine (ACh), which among other things is responsible for the signal transmission between neurons and skeletal muscle cells at the neuromuscular junction. A common classification for dividing neurotransmitters is into amino acids, peptides and biogenic amines even if some neurotransmitters, like nitric oxide (NO) and ACh, fall outside these categories. Amino acid neurotransmitters include two of the most abundant found in our nervous system, γ-aminobutyric acid, or GABA, and glutamate, two classical neurotransmitters that communicate through synaptic, or wiring, transmission.[1] There are two classified types of synapses, symmetric synapses where the pre- and postsynaptic membranes are equally dense and asymmetric synapses where the postsynaptic membrane is denser than the presynaptic one.[6,7] It was suggested early on that symmetric synapses are the main place for inhibitory transmission while excitatory transmission mostly takes place in asymmetric ones. This was confirmed by the finding that GABA, the main inhibitory transmitter in the mammalian brain, is almost exclusively released in asymmetric synapses while the main excitatory transmitter glutamate is released in symmetric synapses.[8] Whereas GABA and glutamate are responsible for classical fast acting transmission, where the neurotransmitter rarely escapes the synapse, other transmitter molecules, like the biogenic amines dopamine, serotonin and norepinephrine, and probably neuropeptides as well, primarily communicate through spill-over of neurotransmitter or release from locations outside the synapse though volume transmission. Some neurotransmitters can be both inhibitory and excitatory, while others are specialized for one type of signaling. Many neurotransmitters have also been reported to act as neuromodulators.[5,9,10] The neurotransmitter that I have studied in my work is dopamine. It is a part of the biogenic amine group of transmitters and will be described in more detail in the following section.

## 1.5. Dopamine

The hormone dopamine was discovered to also function as a neurotransmitter in the brain through a series of studies published in the late 1950s.[11-13] Dopamine has been implicated to be important in addiction and reward and also in many diseases, like schizophrenia, ADHD, and Parkinson's disease, thus it is an extensively studied neurotransmitter. Dopamine is also involved in the control of movement, a connection made clear through the observation of movement impairment in

Parkinson's disease as a result of decreased dopamine levels in the striatum. The finding that treatment with the dopamine precursor L-DOPA decreased these symptoms further strengthened the connection.[1] Dopamine neurons are predominantly located in the midbrain, where some major pathways originate in the substantia nigra, with fibers projecting into the caudate-putamen, and from the ventral tegmental area mainly extending into the nucleus accumbens and the olfactory tubercle.[14] Dopamine largely acts in an inhibitory fashion, but excitatory actions have been reported as well.[15] Inhibitory or excitatory, release of dopamine does not normally seem to elicit neuronal activity directly. Instead this transmitter generally acts though neuromodulation, altering the responsiveness of neurons to stimuli.[14] In accordance with this, dopamine signaling occurs mostly through volume transmission in so called social synapses, where the output of dopamine from one release site reaches tens to hundreds of postsynaptic sites.[9] In fact, experimental findings suggest that dopamine mainly is released immediately outside the synapse and diffuses in the extracellular space affecting receptors over a large area.[9,14] Based on the volume transmission nature of dopamine signaling, the release of this transmitter from neurons has been likened to that of hormones from adrenal cells of the medulla.[16]

There are five subtypes of dopamine receptors in the brain, termed $D_1$-$D_5$, all acting via G proteins. Binding of dopamine to $D_{1A}$-$D_{1D}$ and $D_5$, further grouped as the $D_1$-like receptors, activates adenylyl cyclase, whereas binding to the other subtypes, grouped together as $D_2$-like receptors, causes inhibition of adenylyl cyclase and activation of $K^+$ channels. Following release, dopamine is either metabolized into homovanillic acid (HVA), or taken up into the cell again by the dopamine transporter (DAT). This transporter is mainly located outside of synapses, further strengthening the view of dopamine as primarily acting though volume transmission.[8] Inside the cell, the dopamine molecules can be loaded into vesicles again or metabolized into dihydroxyphenylacetic acid (DOPAC) by monoamine oxidase (MAO).[1]

In order to communicate with surrounding cells, the neurotransmitter needs to be transported out of the cell. No matter which pathway is involved in transmission, almost all neurotransmitter molecules are released from the presynaptic neuron through the same universal mechanism; exocytosis.

# 2. EXOCYTOSIS

## 2.1.  The Exocytotic Process

### 2.1.1. Vesicles

Communication between cells largely relies on the transfer of chemical messengers. These chemicals, also called neurotransmitters, are stored in the presynaptic nerve terminal inside membrane bound vesicles. Most vesicles involved in the release of neurotransmitter in neurons are of a type called small synaptic vesicles (SSV), with diameters of around 40 nm,[1,17] however, there are also larger vesicles called large dense core vesicles (LDCV) present in some neuronal cells.[18,19] These larger vesicles, containing a densely packed protein matrix, are also present in many other cells, like exocrine, endocrine and neuroendocrine cells where they release chemicals through regulated exocytosis.[20] Both LDCV and synaptic vesicles are formed from the *trans*-Golgi network.[21] The formation of LDCVs most likely happens through the aggregation of secretory proteins. Expression of Chromogranin A in fibroblasts has been found to induce the formation of dense core vesicles, whereas down regulation decreases the number of vesicles in PC12 cells. This suggests an important role for this protein in the formation of these vesicles, perhaps through the wrapping of the golgi membrane around the forming dense core.[20] Cholesterol and dynamin II have been suggested to facilitate the scission of the vesicle from the Golgi network. Cholesterol by adding negative curvature to the neck formed or alternatively by recruiting important proteins, while dynamin II has been suggested to play the role of dynamin I in endocytosis; forming an extending helix around the vesicle neck resulting in its breaking.

One important aspect of neurotransmitter containing vesicles is their acidic interior, with a pH of 5.4 compared to the surrounding physiological pH of 7.4. The acidification is accomplished through the action of a $H^+$-ATPase in the vesicle membrane, pumping protons into its interior. This pH gradient is vital since the vesicle normally is loaded with transmitter though the action of an antiport transporter protein present in the vesicular membrane, which transports the neurotransmitter molecules into the vesicle while jettisoning protons. In the case of dopamine and the other monoamines, this protein is called the vesicular monoamine transporter, or the VMAT (VMAT1 in endocrine cells and VMAT2 in neurons) and it is highly efficient at working against a steep concentration gradient. This is necessary since the concentration of cytosolic dopamine needs to be maintained at low levels due to its toxic nature.[22] Furthermore, the efficiency of the VMAT ensures

that a discrete high concentration pulse of neurotransmitter will exit the presynaptic cell following fusion of the vesicle with the cell membrane.

### 2.1.2. Vesicle Recruitment and Fusion

For the vesicular content to reach the synaptic cleft, the vesicle needs to approach and subsequently fuse with the plasma membrane (Figure 2). The protein machinery behind recruitment and fusion of vesicles is highly conserved throughout biology, allowing knowledge extracted from LDCVs to unravel the universal details of the complex exocytotic process. The major players involved in vesicle docking and fusion are the three proteins of the SNARE complex; VAMP (or synaptobrevin), syntaxin and SNAP-25. VAMP/synaptobrevin is located in the vesicle membrane (v-SNARE) and the other two proteins are mainly found in the plasma membrane (t-SNAREs). The SNARE complex is essential for exocytosis, partly evidenced by the fact that many neurotoxins, like the botulinum neurotoxins, act through the cleavage of these proteins.[20] The first step of association with the plasma membrane is called tethering and it is believed to be the determining step for localization of the vesicle to the correct membrane region. A GTPase called Rab, its effector EEA-1, and the SNAREs, as well as other cytosolic factors, are all believed to take part in this process, but the details of membrane recognition and vesicle tethering are not clear.[23-25] The vesicles are then docked to the membrane through the formation of the highly stable SNARE complex. However, there are other proteins involved in the docking step as indicated in part by the fact that application of the VAMP cleaving tetanus toxin to giant-squid nerve terminals results in the accumulation of docked vesicles, also suggesting a function of the SNARE complex after the docking step.[20,25] The efficiency of the SNARE complex has been demonstrated *in vitro* in a liposome model where a single SNARE complex was shown to induce slow fusion.[26] However, studies of exocytotic fusion in mutant cells indicate that fast vesicle fusion *in vivo* requires at least three SNARE complexes.[27] The docking of the vesicles ensures that they are in close proximity to the plasma membrane in case their contents need to be released.

The number of docked vesicles in neurons and neuroendocrine cells is highly variable, perhaps indicating the differences in activity levels and signaling patterns of individual cells. Hippocampal synapses have been reported to contain around 200 vesicles of which 4-10 are in their docked stage, also called readily releasable vesicles, while 17-20 are found in the so called reserve pool which acts as a back-up to the release-ready vesicles. The rest of the vesicles of the terminal have been hypothesized to form a resting pool.[17] In contrast, the motor neurons of the neuromuscular junction have no reported resting pool. Instead, 20 % of the vesicles are readily

releasable and the rest are in the reserve pool.[28] The vesicles in a neuroendocrine cell also exist as two different populations based on their level of maturation and on their physical position, where the size of the ready releasable pool of docked vesicles partly depends on the basal internal calcium concentration.[29] Chromaffin cells contain around 22 000 large dense core vesicles of which 1-2 % are release ready. Recruitment of the reserve pool takes tens of seconds and involves the transportation of the vesicles along the cytoskeleton.[30]
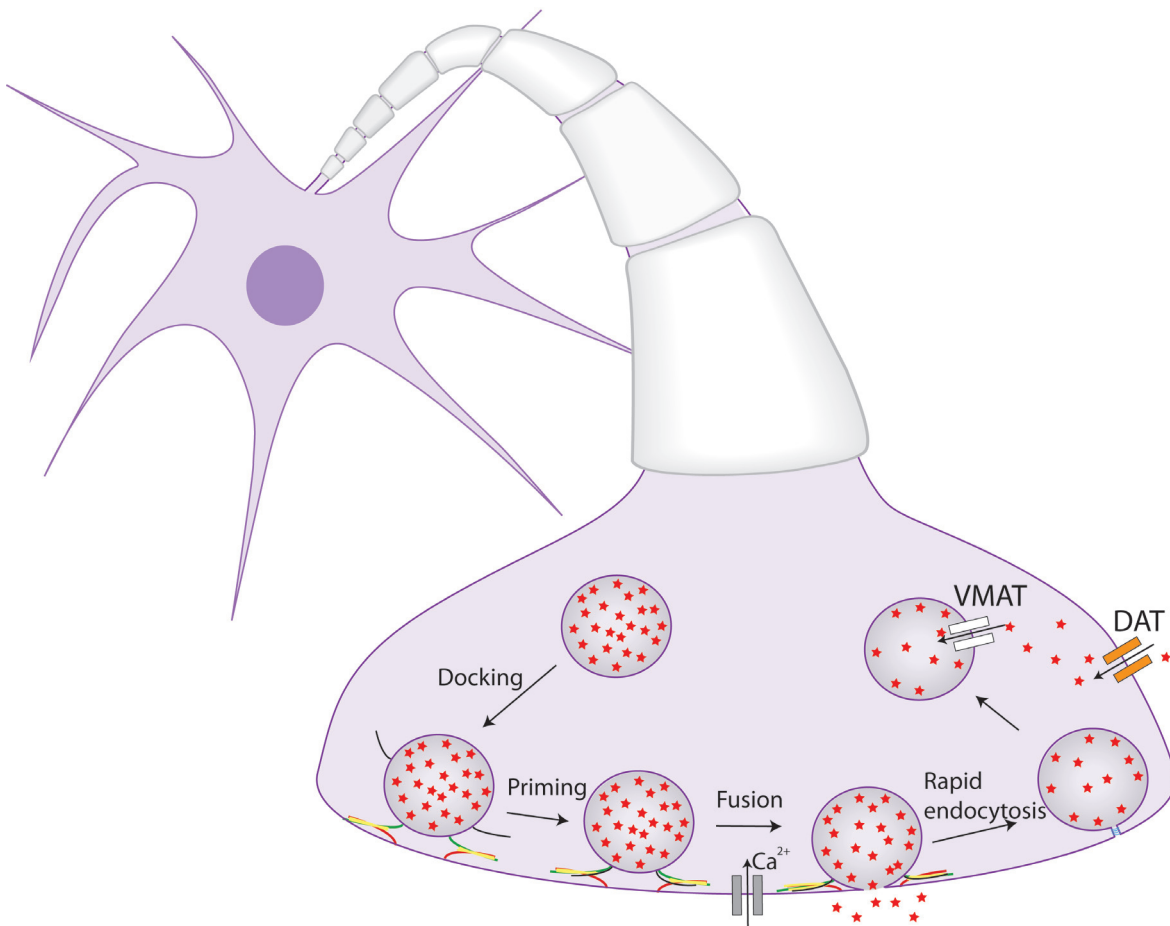


*Figure 2: Schematic of the different stages of exocytosis; docking priming and fusion followed by rapid endocytosis in the presynaptic terminal. The released dopamine is transported back into the cell though the DAT and loaded into vesicles by the VMAT. Dopamine is symbolized by red stars.*

Following docking, the vesicle is prepared for exocytosis though a process called priming, which makes the vesicle responsive to $Ca^{2+}$.[17] When an action potential reaches the synapse, voltage gated calcium channels are opened, resulting in an influx of $Ca^{2+}$ into the cell. Upon the rise in intracellular $Ca^{2+}$ concentration, the SNARE complex pulls the vesicle closer to the membrane. This action is probably mediated though the $Ca^{2+}$ dependence of synaptotagmin, a protein that is abundant in vesicle membranes and that interacts with the SNARE complex. It is believed that synaptotagmin, upon binding of $Ca^{2+}$, is partially inserted into the plasma membrane, an action that is thought to help the induction of bilayer fusion.[17] $Ca^{2+}$ has also been suggested to interact with the lipids of the plasma membrane, neutralizing their charges, making it easier for the vesicle to approach the plasma membrane.[31] In nerve cells, the increase in intracellular $Ca^{2+}$ concentration during exocytosis has been accessed through the use of caged $Ca^{2+}$ and estimated to 5-10 µM during an action potential,[17] an increase that takes seconds to decline to resting levels.[32] However, the flux of $Ca^{2+}$ into the presynaptic cell during an action potential is usually restricted to a small area, resulting in a return to basal levels within milliseconds.

As the calcium ions help evolve the vesicle fusion machinery, the vesicle membrane is brought in close proximity to the plasma membrane, finally inducing their mixing into one common bilayer. This is believed to take place through an initial hemifusion stage, where the inner leaflet of the plasma membrane fuses with the outer leaflet of the vesicle. Following this stage, the inner leaflet of the vesicle fuses with the outer leaflet of the plasma membrane, creating a fusion pore connecting the inside of the vesicle to the outside of the cell, allowing the neurotransmitter to escape. The insertion of the vesicle membrane into the plasma membrane inherently leads to the loss of the vesicle and an increase in the cell membrane surface area. This is prevented by the succeeding process of endocytosis.


### 2.1.3. Endocytosis

The fused vesicle is thought to be retrieved by the cell through a process called endocytosis. There are two suggested modes of vesicle retrieval; rapid endocytosis, which occurs at a time scale of hundreds of milliseconds to seconds, and a slower form of endocytosis that takes tens of seconds to minutes.[33] The slower form of endocytosis retrieves the vesicle membrane after it has been fully incorporated into the plasma membrane. It is mediated though the coating of a membrane patch with a protein called clathrin, forming so called clathrin-coated pits. Vesicles retrieved by this mechanism are easily identified based on their protein coat and studies have shown that their abundance does not change with altered neuronal activity, indicating that this form of endocytosis is not intimately linked to exocytotic release of

neurotransmitter.[34] Instead it seems that rapid endocytosis is the predominant mechanism of vesicle retrieval following stimulated exocytosis.[34,35] In this form of endocytosis, the vesicle is retrieved without collapsing into the cell membrane, a process that involves the actions of the protein dynamin, which is believed to assemble into a spiral around the fusion pore eventually resulting in the fission of the vesicle from the membrane.[36] The primary cellular components involved in the process of vesicle fusion and fission are the membrane of the fusing vesicle and the cell plasma membrane, further explored in the next section.

## 2.2. The Phospholipid Bilayer Membrane and its Role in Exocytosis

The membrane surrounding the cell and its organelles is constructed by a phospholipid bilayer. Fully 5% of the eukaryotic cell genome is involved in the production of thousands of different lipids, resulting in a multitude of phospholipid molecules.[37] These lipids are utilized for energy storage as well as for second messengers, but in this thesis I will focus on the polar lipids that constitute the matrix of the cell membranes. Phospholipids are composed of a hydrophilic head group and a hydrophobic tail in the form of two acyl chains. This allows the formation of a bilayer where the lipophilic tails self-associate and the hydrophilic heads face the surrounding water forming a compartment protected from the surrounding solution. This is a spontaneous process that has implications throughout biology, and has made life possible. The lipid bilayer surrounds not only the cell but also the organelles inside the cell, like the exocytotic vesicles. This lipidic membrane allows for the exchange of material between the cell and its exterior via processes like endo- and exocytosis.

The most abundant lipids in eukaryotic membranes are the glycerophospholipids; phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI) and phosphatidic acid (PA). These lipids have a diacylglycerol (DAG) tail group containing saturated or *cis*-unsaturated hydrophobic acyl chains. The most common glycerophospholipid in the membrane is PC which constitutes over 50 % of the phospholipids, followed by PE which is the second most abundant at 20 %. However, PE is enriched in the brain where it constitutes 45 % of the total phospholipid.[38] A second class of phospholipids is made up of the sphingolipids, like sphingomyelin (SM) and glycosphingolipids (GSLs). These lipids have saturated or *trans*-unsaturated acyl chains giving them a cylindrical structure that is narrower than the structure of PC.

The cell membrane is asymmetric with respect to lipid distribution, with phosphatidylethanolamine (PE) and phosphatidylserine (PS) predominantly in the

inside leaflet and phosphatidylcholine (PC) and sphingomyelin (SM) in the outer leaflet. Also, the lipid distribution in the different membrane compartments of the cell varies and is highly regulated indicating the importance of the specific structure of individual lipid molecules for the functions of the membrane.

One aspect of the lipids in the cells plasma membrane, believed to be important for its functions, is their ability to separate into different phases. The narrow structure of sphingolipids allows them to pack tightly and thereby form a solid gel phase, in contrast to most of the plasma membrane where the lipids are in a liquid disordered state. The solid gel phase is made fluid by the introduction of sterols, like cholesterol, which is the dominant non-polar lipid in the cell. The fluid state formed by sphingolipids in combination with cholesterol is called a liquid ordered phase and this phase is believed to form so called lipid rafts.[37] SNARE proteins are highly enriched in lipid rafts suggesting that these patches of the plasma membrane play a part in localizing exocytotic release.[39] Further supporting this idea, depletion of cholesterol in the plasma membrane of PC12 cells has been found to suppress exocytosis, suggesting a connection between cholesterol enriched rafts and release.[39]

In addition to its implications in localization and recruitment of vesicles, the lipid composition of the bilayer membrane is also deeply involved in the fusion and release process of exocytosis. High curvature lipids have been shown to completely arrest exocytosis,[40] alter the kinetics and efficiency of release[41-43] and to influence the dimensions of the initial fusion pore,[42-44] indicating the importance of membrane lipid composition in the exocytotic process. The dependence of exocytosis on membrane composition is most likely caused by the presence of very high curvature regions during the process, making some curvatures favor, and others inhibit, fusion. The head groups and the tails of the phospholipids differ, and the size of the head group in relation to the size of the tail gives the molecule a distinct shape. Because of a rather large choline head group, PC has a near cylindrical shape while PE, with its relatively smaller ethanolamine head group, has a conical shape. Lipids with head groups that are larger than the tail are referred to as inverted cone shaped. One such lipid is lysophosphatidylcholine (LPC), which has a PC head group but only one tail. These phospholipid shapes have different intrinsic curvatures, meaning that a membrane made up of cylindrical lipids will be flat, while a membrane made of cone, or inverted cone, shaped lipids will be curved (Figure 3).
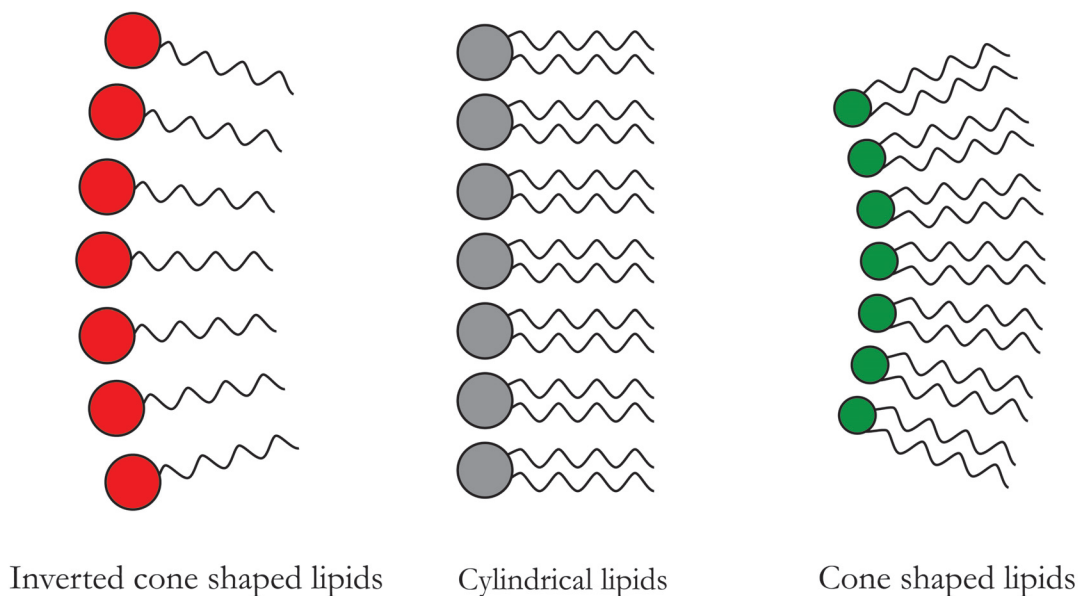
Inverted cone shaped lipids    Cylindrical lipids    Cone shaped lipids

*Figure 3: The intrinsic curvature of the three different lipid shapes as demonstrated by their spontaneous formation of curved monolayers.*

Introducing PE into a PC bilayer induces curvature in the membrane, a property that is explored during high curvature processes like budding, fission and fusion. PE has for example been shown to be enriched in the high curvature regions of mating *Tetrahymena* using imaging mass spectrometry.[45,46] In the studies of lipid distribution in *Tetrahymena* it was also shown that the segregation of PE into the pores formed during mating was induced by the formation of high curvature regions. Lipid sorting based on membrane curvature has also been demonstrated using molecular dynamic simulations.[47] Another aspect of lipid composition in relation to curvature is the symmetry or asymmetry between the membrane bilayers, where asymmetry can induce curvature stress.[37] Evidently, the presence of high curvature lipids influences the behavior of lipid membranes in high curvature structures and affects the process of membrane fusion in exocytosis (Figure 4). These implications are further explored in paper III and IV of this thesis.
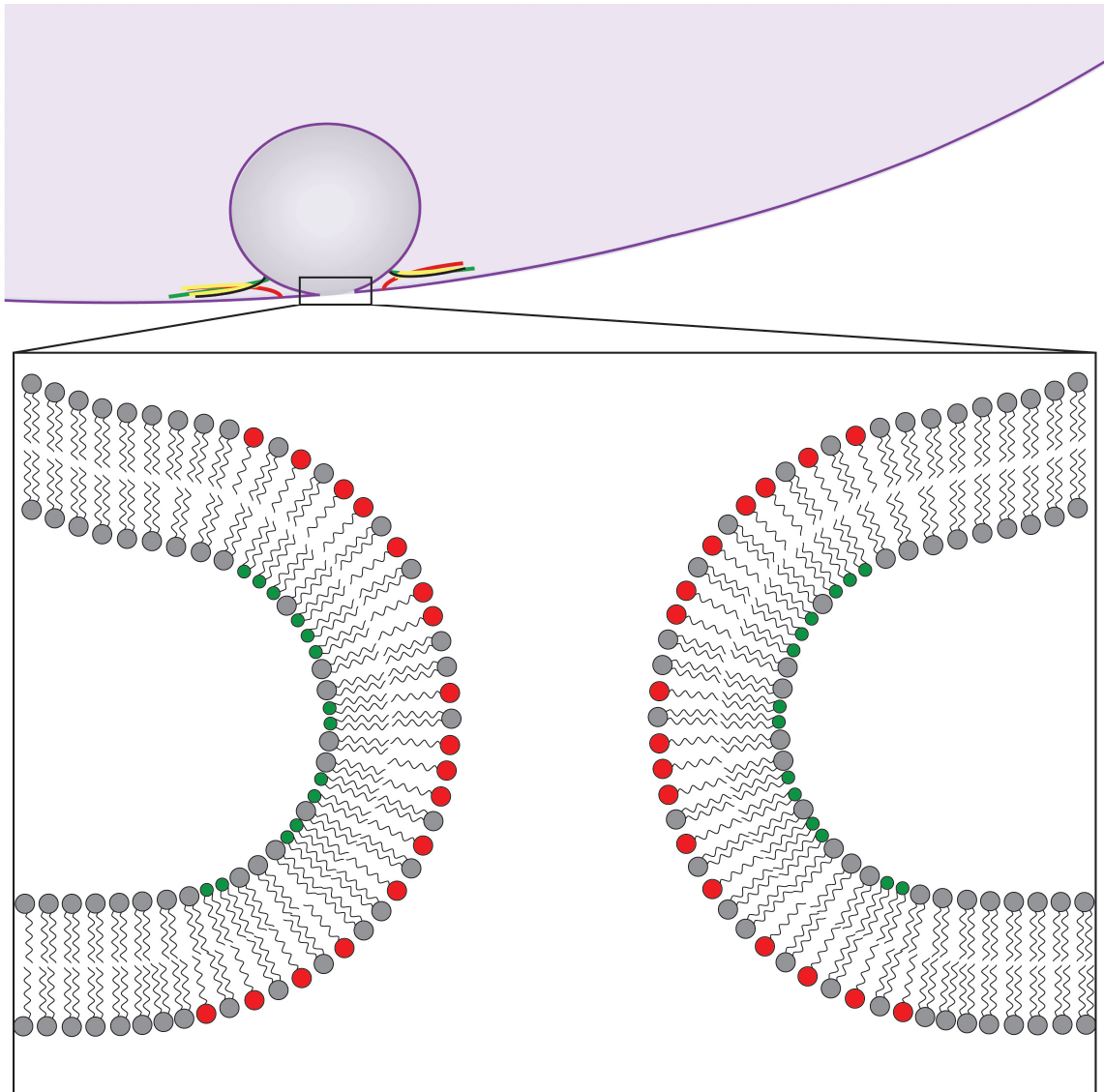
*Figure 4: The high curvature regions formed during exocytosis are influenced by the lipid composition of the membranes involved. In the initial fusion pore, the inner leaflet has a curvature that is favored by inversely cone shaped lipids like LPC, while the outer leaflet prefers cone shaped lipids like PE.*

## 2.3. The Quantal Nature of Neurotransmitter Release

The quantal nature of neurotransmission was first observed by Fatt and Katz in 1952.[48] While studying the end-plate potential of muscle fibers using an intracellular microelectrode to measure the membrane potential, they noticed small potential spikes in innervated muscle areas coinciding in space and time with the initiation of the end-plate potential. These discharges had average amplitudes of 0.5 mV, which is about 1/100 of a normal end-plate potential. They also introduced a relationship between the action potential and the release of neurotransmitters, suggesting that the discharges were the result of some reaction between released ACh and the postsynaptic cell.[48,49] They proposed a model where the neurotransmitter is released at specific sites, resulting in a quantal fashion of release. In 1955, De Robertis and Bennet studied synapses from the earthworm with the aid of electron microscopy. They observed vesicles in the presynaptic nerve terminals and they suggested that these vesicles were packed with neurotransmitter which was released from the cell through some sort of perforation by the vesicles through the membrane.[50] From this seminal work sprung the idea of exocytosis as an all or none process, meaning that if a vesicle fuses it is going to completely collapse into the membrane expulsing its entire inner volume to the exterior of the cell (Figure 5) and that the full content of the vesicle makes up the quanta.

The idea of complete vesicle collapse and release has however come to be revised into a more complex one, initially based on the observation that vesicles can fuse in a transient manner. The first observations of transient vesicle fusion were made with patch clamp measurements alone,[51] and when combined with amperometric detection it was concluded that neurotransmitter was released during the transient events.[52] Subsequent studies on neurons[53-55] and neuroendocrine cells[56,57] have strengthened the view of transient fusion, also termed kiss-and-run, as an important mode of release (Figure 5). In fact, recent studies suggest that kiss-and-run fusion is the predominant form of exocytosis in chromaffin cells[56,58] and neuronal cells,[53,59,60] however, the mode of release has been indicated to vary with the stimulation conditions.[56,58,61,62] In paper III of this thesis we introduce the concept of extended kiss-and-run, where the vesicle forms an initial fusion pore whereafter the pore expands transiently. This is followed by the reformation of the fusion pore and the subsequent resealing of the vesicle (Figure 5). Kiss-and-run fusion makes the quantal nature of exocytosis more complicated, where the size of the quanta is determined both by the amount inside the vesicle and by the fusion process.[63]
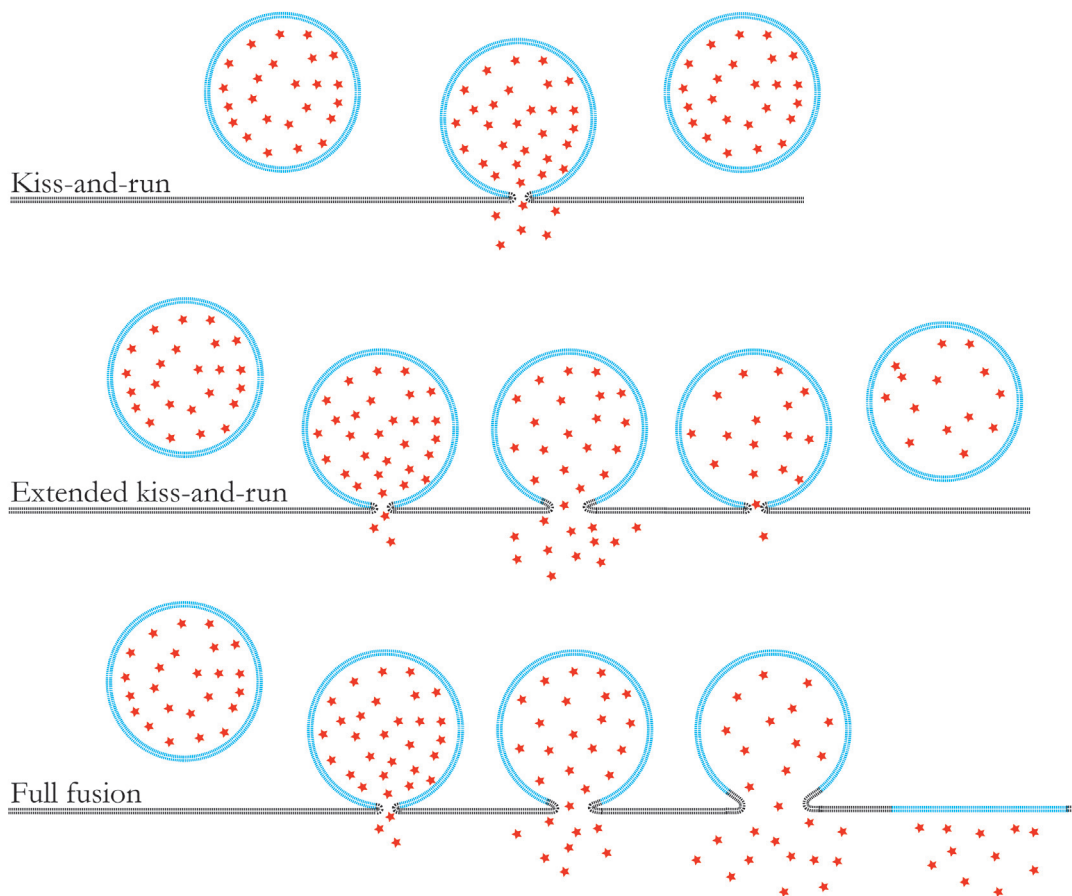
*Figure 5: Proposed modes of exocytosis. The vesicle membrane is blue and the plasma membrane is black. The neurotransmitter is symbolized by red stars.*

## 2.4. Do Vesicles Release Their Full Content During Exocytosis?

If vesicle fusion is transient and the vesicle doesn't completely collapse into the plasma membrane, there is a possibility for incomplete release of the vesicular neurotransmitter content. The most direct evidence for fractional release comes from studies on PC12 cells where the use of a method called electrochemical cytometry made the analysis of the neurotransmitter content of single vesicles possible.[64] Comparison between the amount in the vesicles and the amount released as measured with amperometry reveals that fractional release is the most abundant form of exocytosis in these cells. The average fraction of the content released has been calculated to be 40 %.[65] Further supporting the idea of fractional release of neurotransmitter, there are many factors that have been shown to modulate the amount of neurotransmitter released in fashions that most likely do not affect the

number of molecules contained in the vesicle prior to fusion. For example, the lipid composition of the membrane can decrease or increase the amount released after very short incubations, an experimental scheme that most likely results in the confinement of the introduced phospholipid to the outer leaflet of the plasma membrane. This effect was first observed in chromaffin cells and has thereafter been demonstrated in PC12 cells as well.[42,43] The osmolality of the solution surrounding the cell has been shown to alter the amount of transmitter released per event from chromaffin cells, where a hypertonic solution decreases the amount released and a hypotonic one increases it.[66-69] The use of patch-amperometry has revealed that the number of molecules released per event is significantly larger in this mode than when monitored with amperometry alone, indicating that the pressure applied to the membrane by the patch pipette increases the fraction released.[70,71] Some recent studies on neuronal cells also support the idea of fractional release. Synaptic vesicles in hippocampal dopamine neurons in culture have been shown to primarily release transmitter through a narrow fusion pore that repeatedly opens and closes in a so-called flickering fusion. This study also revealed that the simple events that were observed (not flickers) most likely release only about 30 % of the vesicular content.[53] Furthermore, a recent publication analyzing the dopamine content of synaptic vesicles isolated from the mouse striatum showed an average vesicle content of 33 000 molecules.[72] This is a number far exceeding the amount of neurotransmitter normally detected during release events from neuronal cells.[73,74] Together, these pieces of evidence indicate that the full neurotransmitter content of single vesicles is not expelled during an average release event from neuroendocrine or neuronal cells.

## 2.5. Biological Reasons for Transient Fusion and Partial Release

There are important implications for partial release of neurotransmitter in regards of neuronal communication. The level of neuronal activity governs brain and bodily functions, and this activity is not static. The plasticity of neuronal communication can be divided into pre- and postsynaptic mechanisms.[63] Suggested pathways of presynaptic plasticity include alteration of the calcium influx upon the arrival of an action potential, alteration of the probability of release at a given calcium concentration and alteration of the amount of transmitter released per vesicle.[17,22,75] The amount of transmitter released per vesicle has traditionally been thought to be solely determined by the amount of transmitter inside the vesicle prior to fusion. However, the idea of partial release provides a new possibility where the amount expelled can be regulated in the fusion process by altering the fraction released.

Monoamine transmitters, as well as ACh and neural peptides, seem to primarily communicate though volume transmission. Consequently, their receptors are rarely, if ever, saturated.[22,76] Since they are not saturated, increasing or decreasing the released quantal size, whether this is via a change in vesicle content or the fraction expelled, will have an effect on the number of receptors that are activated though the fusion of a vesicle, and also on how long the receptor will be activated since a larger number of molecules will take a longer time to clear.[9,76] The idea of sub-saturation levels of neurotransmitter might also be relevant in private synapses, as indicated by the large variability in quantal sizes release at these synapses.[76] This suggests that postsynaptic receptors are not always saturated by the amount of transmitter released from a single vesicle.[22] In further support of this idea, increasing the concentration of glutamate outside a neuron has been shown to increase the postsynaptic quantal size, providing evidence that the receptors at that synapse are normally not saturated during neuronal activity.[77]

Also related to release being affected by the mode of fusion, it has been suggested that catecholamines and neuropeptides can be selectively released from chromaffin cell granules by tuning the size of the fusion pore in response to the intensity of stimulation.[78] In addition to the implications of the regulation of released amount in neuronal plasticity, the extent and rate of fusion pore expansion has been suggested to play a role in long-term potentiation in hippocampal glutamate silent-synapses.[79] These synapses express high-affinity NMDA receptors and silent low-affinity AMPA receptors. The AMPA receptors become responsive following LTP, and it has been shown that this activation can occur though the increase of peak glutamate concentration in the synapse as a result of altered fusion pore expansion rates.

Transient vesicle fusion has other advantages in addition to the implications of partial release in synaptic plasticity. There is a purely energetic gain in not completely collapsing the vesicle lipids into the membrane in the sense that you save the energy and time of retrieving the vesicle.[54] Also, if the vesicle is collapsed into the cell membrane, its components will be mixed in with the plasma membrane and there will be a need to sort out this complex set of lipids, proteins and sugars again, adding to the energy saving advantages of kiss-and-run fusion followed by rapid endocytosis.[34] In the case of dense core vesicles, one reason for the vesicle to not completely collapse into the plasma membrane might also be to not release the dense core proteins. This will of course keep the protein from entering the extra cellular fluid and being lost, but also avoids the requirement for production of a new dense core, a clear advantage from an evolutionary standpoint since the cell is most likely not able to synthesize protein in the nerve terminal.[1]

It seems that there are many advantages for the cell in employing transient vesicle fusion with partial release of the vesicle content. However, it is still being debated whether neurotransmitter release primarily occurs though full or partial distension of the fusion pore. To gain further insight into the vital process of exocytosis, the availability of analytical tools with the ability to resolve single release events will be essential. The following chapter will discuss techniques presently available for such studies.

# 3. STUDYING EXOCYTOSIS

## 3.1. Methods for Studying Single Release Events

The study of single release events represents a formidable analytical challenge. The size scale of the vesicle ranges from 20-150 nm in radius while the fusion pore is in the range of a few nm, well below the limits of traditional optical microscopy. Individual release events are also ephemeral, lasting less than a millisecond in the case of synaptic vesicles to several milliseconds in neuroendocrine cells. High sensitivity is also vital since a single release event results in the expulsion of between millions of molecules for some neuroendocrine cells down to thousands of molecules from neurons.[80] Thus, investigations of single release events require analytical techniques that push the limits of spatial and temporal resolution while maintaining high sensitivity.

### 3.1.1. Imaging

The importance of imaging in biological research cannot be overstated. Although as mentioned above, to image vesicles fusing with a cell membrane, you need an imaging technique with extraordinary spatial resolution. There are two methods that meet the criteria by creating sub-diffraction limited illumination of the sample. These are total internal reflection fluorescence microscopy (TIRFM) and stimulated emission depletion (STED). TIRFM takes advantage of the quickly decaying evanescent wave created when the incoming light beam is totally reflected at the interface between a cover slip and the sample.[81] For imaging with STED, two lasers are used, one that excites the sample and a second laser that de-excites it. The second laser is shaped like a doughnut around the first laser, reducing the resultant field of excitation.[82] The small size of the excitation field results in low background signals, allowing the acquisition of images of single vesicles close to the plasma membrane inside a cell.[83-86] These imaging techniques cannot, however, be used without some form of fluorescent labeling of the vesicle. Available strategies for loading the vesicle with dye include, but are not limited to, labeling of the vesicles interior membrane through the uptake of dye via endocytosis, diffusion of dye into the vesicle that is trapped when protonated in the acidic environment and genetic modification of vesicle components to express some fluorescent protein.[87] The super resolution imaging techniques allow one to monitor individual vesicles inside cells, providing information on vesicle movement, docking and fusion. However, the temporal

resolution is not sufficient for detailed studies of the stages of an exocytotic release event, and the addition of dye molecules creates an unnatural state in the system.

### 3.1.2. Electrophysiology

As previously mentioned, the use of electrophysiological techniques allowed Katz and coworkers to notice that the action potential in the frog neuromuscular junction was built up from smaller individual pulses eventually leading to the identification of quantal release of neurotransmitter.[48,49] These types of electrophysiological measurements, where the potential over a membrane is monitored, are widely used in the study of neuronal activity, since they allow the monitoring of neuronal activity in pre- and postsynaptic cells. However, for the study of single exocytotic events, the electrophysiological method of choice is called patch clamp. The patch clamp technique was first developed as a tool for studying ion channel conductance,[88,89] and later its potential for monitoring exocytosis was realized.[90]

The technique involves the creation of a tight seal between the cell membrane and a micropipette (Figure 6). This setup can be used to measure membrane conductance by clamping the potential across it while monitoring the current though it, allowing the detection of a single ion channel opening.[88] In addition to being sensitive to currents though the membrane, the patch clamp technique can be adjusted to study membrane capacitance, which is directly related to the size of the membrane. Since the fusion of a vesicle with the cell membrane increases its surface area this will lead to an increase in membrane capacitance. To study single exocytosis events with patch clamp, a sine wave potential is applied over the membrane. The capacitance of the membrane can then be estimated from the phase shift of the resultant current.[91]

In addition to exocytosis, this method can be used to monitor endocytosis since this process is accompanied by a decrease in plasma membrane surface area. The patch clamp technique is a powerful technique for monitoring fusion and fission of vesicles with the plasma membrane with high temporal resolution. However, it is not sensitive to the content released during the fusion event, leaving the amount and the identity of the chemicals released unknown.
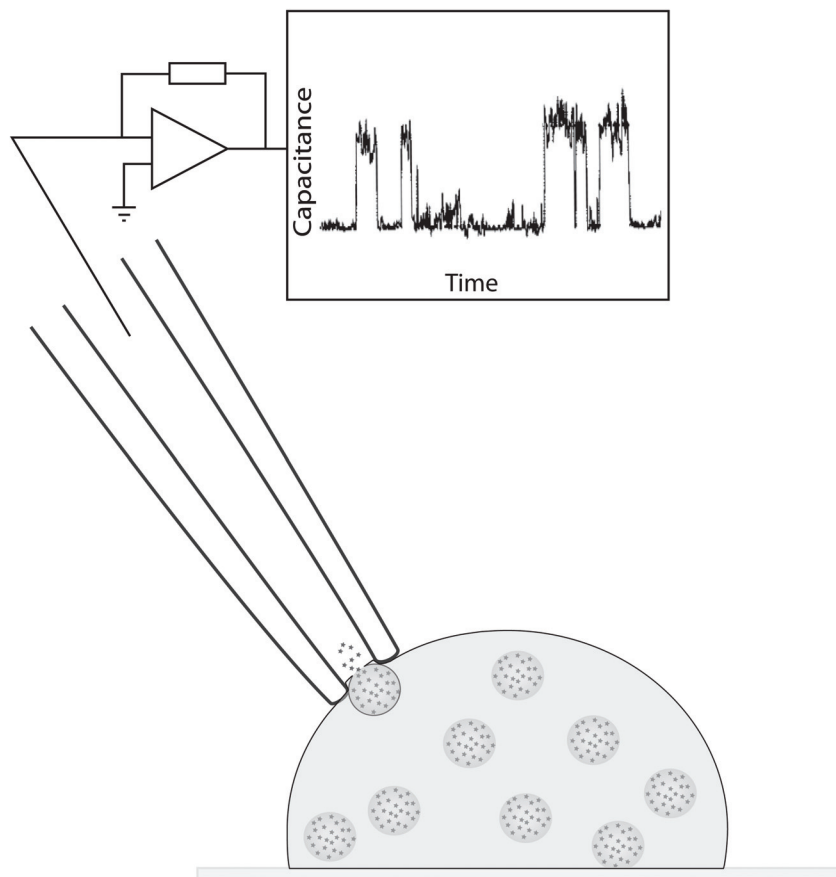
*Figure 6: Schematic of the patch clamp technique applied to the study of exocytotic release. The fusion of a vesicle with the cell plasma membrane results in a capacitance step. The example trace has been modified from reference 57.*

### 2.1.3. Electrochemistry

The electrochemical nature of many neurotransmitters, like the catecholamines, permits the use of electroanalytical methods in the study of exocytotic release.[92,93] There are two electrochemical methods widely used for this purpose, amperometry and fast scan cyclic voltammetry (FSCV) (Figure 7). Both methods make use of a working electrode and a reference electrode, where the reference electrode ensures that the desired potential is applied at the working electrode. The most widely used working electrode for studying exocytosis is the carbon fiber microelectrode that is constructed from a micrometer sized carbon fiber and a glass capillary. Advantages of these electrodes include small size, biocompatibility, flexibility and resistance to strain.[94,95] In paper II of this thesis, two different geometries of carbon electrodes for amperometric detection of exocytosis are compared.
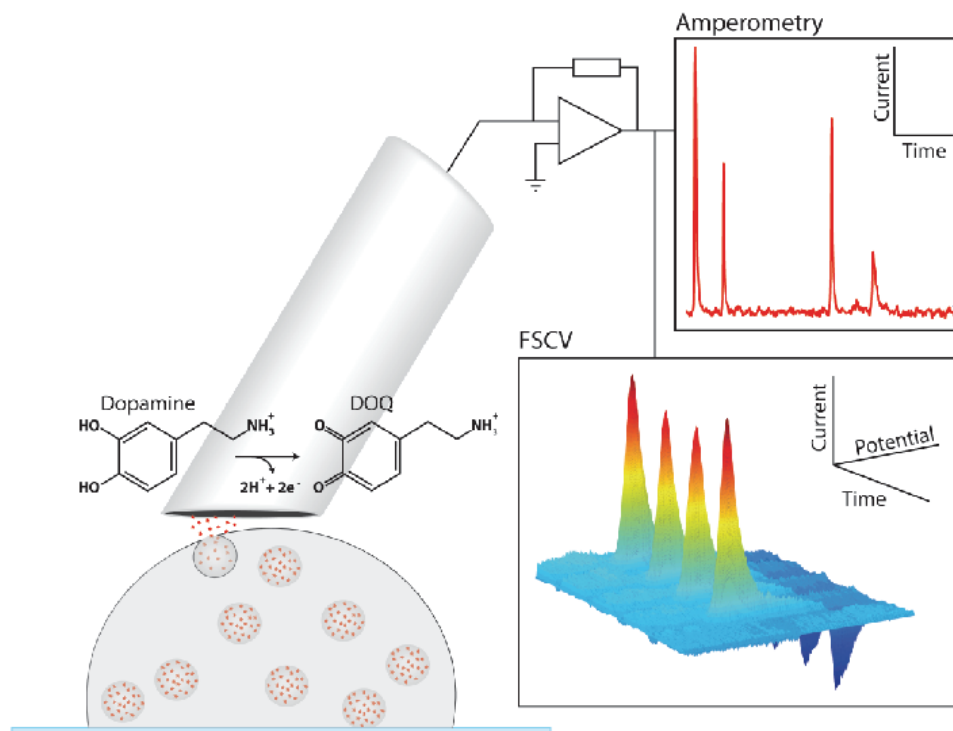
*Figure 7: Schematic of the electrochemical detection of release of dopamine from a neuroendocrine cell with examples of resultant traces for the two methods FSCV and amperometry. The oxidation of dopamine to its reduced form dopamine ortho-quinone (DOQ) at the electrode surface results in current peaks in the amperometric trace. In FSCV, the DOQ is re-reduced following its oxidation resulting in the collection of multiple cyclic voltammograms.*

In FSCV the potential is scanned past the oxidation potential of the molecule of interest and then back again, resulting in a cyclic voltammogram displaying the resultant oxidative and reductive currents in relation to the potential applied. This method is most commonly used in neuroscience to monitor fluctuations of neurotransmitter levels in the extracellular fluid of the brain since the magnitude of the oxidation and reduction peaks is dependent on the analyte concentration. One great advantage in this type of experiment is the non-consuming nature of the method, leaving the neurochemical composition of the extracellular fluid basically intact. FSCV also allows one to distinguish between molecules that display different electrochemical behaviors and has for example been used to discriminate between chromaffin cells that release epinephrine and cells that release norepinephrine, or

both.[96] The time resolution of the method depends on the scan rate and the waiting time between scans. Pushing the limits make the detection of single release events from neuroendocrine cells possible.[97,98] FSCV is, however, not quantitative and the peaks are broadened. This obscures the kinetic information obtained and makes singling out faster release events, like the ones from neurons, problematic.

Amperometric detection, in contrast, offers excellent temporal resolution and quantitative information making it a preferred method for the study of single exocytosis events with carbon fiber electrodes. By positioning a microelectrode at the site of release and holding it at a constant potential where the oxidation of the neurotransmitter is diffusion limited, the molecules that exit the cell through exocytosis can be detected electrochemically.[93,99] The method was pioneered by the Wightman group,[93] and has since been extensively applied to neuroendocrine cells[99-101] but has also been used to study exocytotic release from primary cultured neurons,[53,74,80] brain slices[102] and *in vivo* from intact neurons.[103,104] The time resolution of this technique is limited only by the sampling frequency, which can be adjusted according to the system studied, thus allowing detection of single fast release events. The fusion of a vesicle with the plasma membrane and subsequent release of the electroactive neurotransmitter content gives rise to a current spike.

The method is quantitative through the conversion of the charge passed at the electrode surface into the number of molecules that have been oxidized. This is accomplished through the use of Faradays law;

$$Q = nNF$$

where $Q$ is the charge in coulombs, $n$ is the number of moles of electrons transferred per mole of molecules oxidized (2 in the case of dopamine), $F$ is the Faraday constant (96,483 coulombs/mole of electrons) and $N$ is the number of moles of analyte detected. In addition to the quantitative information, the amperometric spike provides kinetic information about the release event (Figure 8). The half with ($t_{1/2}$), which is the width of the peak at half its maximum amplitude, is a kinetic indicator of the whole release event. The rise time ($t_{rise}$) of the peak gives information about the rate of pore expansion and the fall time ($t_{fall}$) provides indications on rate of diffusion intermixed with a possible closing of the fusion pore. The maximum current ($I_{max}$) is a measure of the maximum flux of neurotransmitter during the release event.
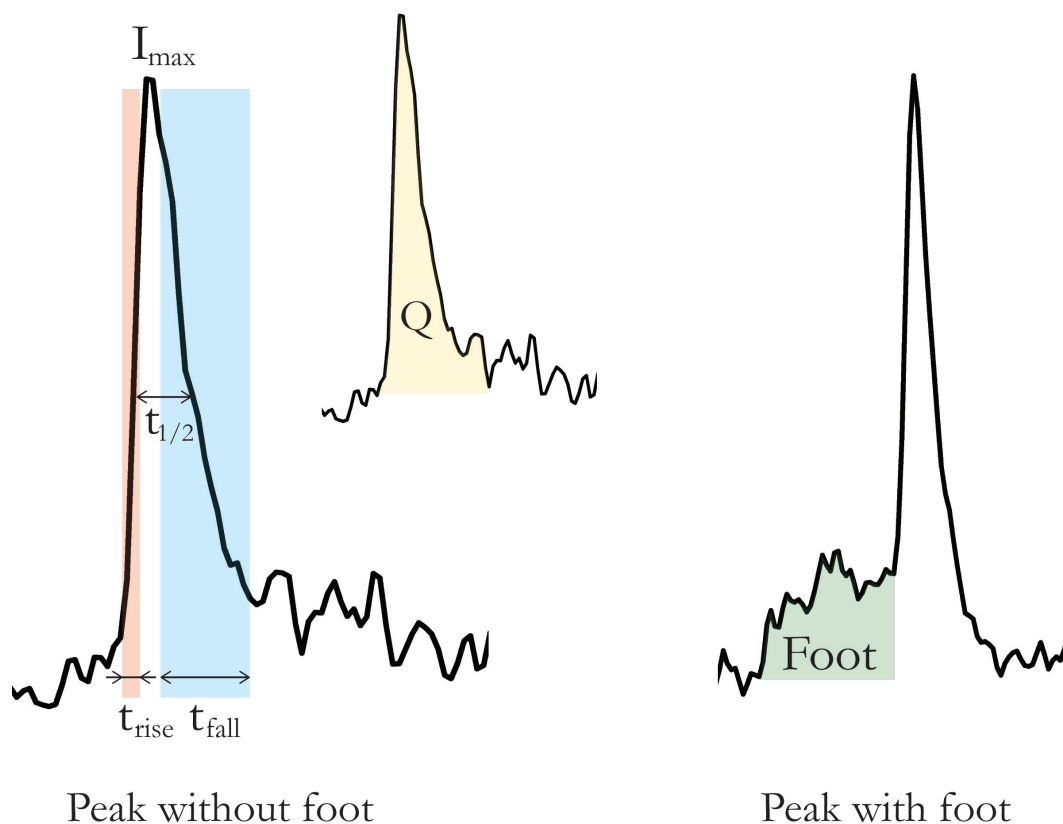
*Figure 8: Amperometric peaks with the peak characteristics typically used for analysis.*

In addition to these peak characteristics, some release events display a small increase in current prior to the peak. This current increase has been termed the foot of the event and stems from the efflux of neurotransmitter though a stable fusion pore prior to its expansion.[105] The height of the foot is dependent on the flux of molecules through the pore which in turn depends on the concentration gradient across the pore and the dimensions of it, i.e. length and diameter. During amperometric measurements, the concentration gradient is equal to the concentration of neurotransmitter inside the vesicle to zero at the electrode. This is caused by the immediate oxidation of molecules as they hit the electrode surface.

Compared to FSCV, amperometry has the disadvantage of not being qualitative in the sense that it does not discriminate between different molecules that are oxidized at the same potential. As a group, electrochemical techniques are limited to the study of electroactive neurotransmitters, although the study of non-electroactive molecules can be approached through the use of enzyme modified electrodes.[106] A second disadvantage of electrochemical techniques is the lack of spatial resolution, which is limited by the size of the electrode. However this aspect

has been approached through the construction of electrode arrays[107-109] and the monitoring of release from both the top and the bottom of a cell simultaneously.[110] In paper I of this thesis, the use of amperometry and FSCV for electrochemical imaging at electrode arrays is compared.

## 2.3. The PC12 Cell as a Model for Exocytosis

The chromaffin cell, which can be isolated from the adrenal medulla and used in primary culture, is the most extensively used system for studying single exocytotic release events. These cells release the hormones adrenaline and noradrenaline from large dense core vesicles into the blood stream, where their actions include regulation of cardiac output, enteric activity, and vascular tone.[111] An alternative system to the chromaffin cell for monitoring single vesicle release events is the PC12 cell. This cell line was established in 1979 from a rat adrenal pheochromocytoma, a tumor originating from a chromaffin cell of the adrenal medulla.[112] The PC12 cell releases dopamine and sometimes noradrenaline from large dense core vesicles (LDCV) and ACh from small clear vesicles through exocytosis in response to an increase in intracellular $[Ca^{2+}]$.[113,114] In response to nerve growth factor (NGF), PC12 cells stop dividing and form neuronal-like protrusions, a process that is reversible upon the removal of NGF.[112] It has been suggested that PC12 cells, based on their NGF induced differentiation have pluripotent qualities and can develop either into chromaffin cells or sympathetic neurons.[112] Differentiated PC12 cells display many similarities to cultured primary neurons with long and highly branched processes. Along the protrusions are specialized regions called varicosities from where the differentiated cell releases dopamine through exocytosis. These events are similar to the release events from undifferentiated cells in that the same average amount of dopamine is released; however, stimulation of varicosities results in fewer release events and a more narrow distribution of the amount released.[115]

PC12 cells express both nicotinic and muscarinic ACh receptors that both, when activated, stimulate release. The nicotinic receptor is connected to a sodium channel that opens upon binding of ACh, resulting in a depolarization of the membrane and subsequent opening of voltage gated calcium channels. Activation of the muscarinic receptor on the other hand leads to a second messenger cascade that releases calcium from intracellular stores.[116] Exocytotic release from PC12 cells can also be stimulated via direct depolarization of the membrane by adding an elevated concentration of KCl to the cell exterior. Depending on the method of stimulation, the time delay between application of stimulant and release varies markedly, where

application of potassium leads to the shortest delay, and stimulation through muscarinic receptors results in the longest one.[117]

There are many similarities between release from neurons and from the neuroendocrine chromaffin and PC12 cells in that they are both $Ca^{2+}$-dependent and carried out by the same proteins, and release occurs in the same steps of docking, priming and fusion. The neuroendocrine cell is a suitable alternative to neuronal cells for studying $Ca^{2+}$-dependent exocytosis since the release takes place over the entire cell body making it easily accessible. Also, the number of molecules released per event is significantly greater. One difference between exocytosis in neuroendocrine cells compared to neuronal cells has traditionally been thought to be a lack of active zones of release.[111,118] However, it has been demonstrated that chromaffin cells display an asymmetric influx of $Ca^{2+}$ upon stimulation and that exocytotic release is localized to regions of high $Ca^{2+}$ concentration.[119] Release hot spots have also been demonstrated in PC12 cells, indicating a presence of active zones in these cells as well.[107,108]

Amperometric detection of exocytosis from PC12 cells was first performed by the Ewing group, from undifferentiated cells in 1994[99] and from varicosities of differentiated cells in 1996,[115] and has since provided valuable insights.[120-123] Compared to chromaffin cells, the PC12 cell has smaller vesicles. The radius of a PC12 cell vesicle is 75-120 nm, whereas chromaffin cell vesicles have an average radius of 170 nm.[116] The smaller size of PC12 cell vesicles results in a system with release amounts and kinetics that more closely resembles those for release from neurons. The time constants for release from neurons is reported to be 0.2-1.2 ms, from PC12 cells 20-200 ms and in chromaffin cells as long as 150-1000 ms.[124] Furthermore, the ability of PC12 cells to form neuronal like outgrowths that release neurotransmitters at varicosities also add to their advantage as a model for synaptic exocytotic release.

## 2.4. Artificial Cell Models

The complexity of the exocytotic process makes studying the influence of isolated proteins or other factors in cells arduous. An alternative approach is the use of artificial cell models where the possibility to minimize and dictate the complexity makes it an attractive system. The cell models used for studying membrane fusion are usually lipid based, taking use of giant unilamellar liposomes (GUVs)[125] or planar lipid bilayers[126,127] to mimic the plasma membrane and small unilamellar liposomes (SUVs) in place of vesicles. Many important aspects of vesicle fusion have been studied using liposome-based models including the SNARE complex,[26] the role of osmotic

forces,[128] and the effect of calcium on spontaneous bilayer fusion.[31] One of the more elegant cell models for studying exocytosis available today was recently developed in the Cans laboratory based on previous studies of DNA-zipper driven vesicle-vesicle fusion.[129] The model consists of a GUV to which a DNA construct has been added to the inner leaflet of the bilayer. Small unilamellar liposomes, with a complimentary DNA construct incorporated into the outer bilayer and filled with catechol, are injected into the giant liposome whereby the two complimentary DNA strands can hybridize, docking the vesicles to the artificial plasma membrane. Fusion between the vesicle and the liposome membrane can then, excitingly enough, be induced by the introduction of $Ca^{2+}$ to the interior of the artificial cell, mimicking exocytosis in living cells. Since the vesicle is packed with electroactive material, the fusion process can be precisely monitored with amperometry outside the artificial cell.[125]
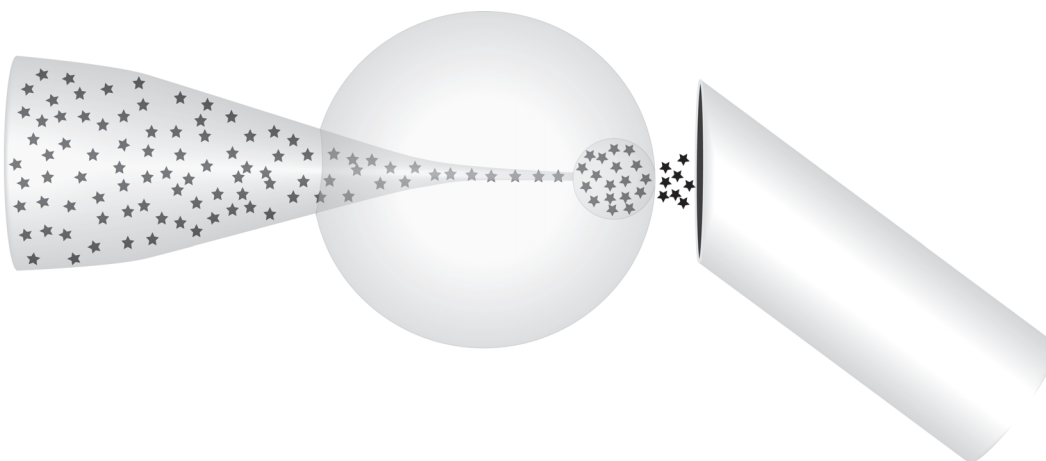


*Figure 9: Schematic of an artificial cell model for exocytosis constructed from a giant unilamellar vesicle. A nanotube is pulled from the giant vesicle and a daughter vesicle is formed through inflation with a solution of electroactive molecules as symbolized by black stars.*

An alternative approach to studying exocytosis artificially was developed in collaboration between the Ewing and Orwar groups.[130] This model consists of a GUV attached to a multilamellar liposome, anchoring the GUV to a surface and acting as a lipid reservoir. A micropipette filled with a solution of some electroactive substance is electroporated through both bilayers of the liposome. As the pipette is pulled back into the liposome it brings with it a lipid nanotube, connecting the pipette to the exterior of the liposome. By applying pressure though the pipette, a

daughter vesicle is formed inside the GUV. The vesicle is expanded until it reaches the membrane of the GUV. This leads to the complete distension of the nanotube and release of the vesicle content in a mimic of the later stages of the traditional view of full fusion exocytotic release (Figure 9). This cell model, employed in paper IV and V of this thesis, provides an excellent system for isolated studies of the membrane dynamics of exocytosis.

# CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The process of exocytosis is the universal method of communication between nerve cells making it a vital one to understand. In this thesis I have provided evidence for transient fusion and partial release of neurotransmitter as the primary mechanism of exocytotic release from PC12 cells. This was done through the identification of a marker of vesicle closing, the post spike foot. Furthermore, I introduce a method for studying fractional release though partial distension of the initial fusion pore in an artificial cell model. The identification of the post spike foot and the establishment of this model for studying partial release open up a multitude of possibilities for future experiments.

By exploring the factors that are believed to affect the fraction of neurotransmitter released during exocytosis, the post spike foot can be further characterized. One such factor is the pressure applied to the membrane when the cell is submitted to suction during patch clamp measurements. The seal of the membrane in the patch pipette has been demonstrated to increase the quantal content released significantly. It would be interesting to see if it is still possible to observe post spike feet during these experimental conditions. A second example of a factor that has been suggested to affect the released fraction is the osmolarity of the surrounding solution, which has been hypothesized to influence the rate of dissociation of catecholamines from the dense core of secretory granules. By bathing PC12 cells in a hypertonic solution, the quantal content of release can be decreased. This would most likely result in a smaller change in post spike foot current compared to pre spike foot current when compared to control cells as there would be more content left for the post spike foot.

The artificial cell model for fractional release through partial distension has numerous potential applications. By altering the lipid composition of the membrane, the direct effects of high curvature lipids on the structures formed during the exocytotic process can be studied. Previous studies have shown that the inverted cone shaped lipid lysophosphatidylcholine increases the rate of release, whereas the cone shaped arachidonic acid decreases it. Incubating the plasma membrane vesicles with lipid containing solutions will result in the incorporation of these lipids into the membrane. In this way the influence of the lipid membrane components on the two different release modes can be studied. Hypothetically, the modes will be affected differently based on the distinct membrane structures that they are constructed from. By comparing results from these experiments to the findings in cells it might be

possible to identify the membrane structures that most resemble the conditions that exist during exocytosis in the cell.

It is obvious from the results in paper V that the kinetics of release is distinctly different for the case of partial versus full distension. These variations in release kinetics could potentially be used to distinguish between different release modes in cells. To explore this, release events from cells stimulated at different frequencies could be studied. It has been suggested from patch clamp measurements that at higher stimulation frequencies, cells more commonly release neurotransmitter through full fusion and at lower rates of stimulation fusion is mostly transient. Hypothetically, one might expect to observe a shift from low amounts released and slow release kinetics at low frequency stimulation towards a higher amount released with faster release kinetics during high frequency stimulation.

In conclusion, the results and methods presented in this thesis can potentially be used to gain further insight into the mechanisms of neurotransmitter release through exocytosis.

# SUMMARY OF PAPERS

The general aim of this thesis has been to further our knowledge of the exocytotic process using amperometric detection of neurotransmitters at carbon fiber microelectrodes. The thesis has two main parts. In papers I-III, different aspects of exocytotic release from PC12 cells are examined. In papers IV and V an artificial cell model is explored to investigate the membrane structures formed during exocytotic release.

In paper I the use of electrode arrays by performing FSCV at the individual electrodes is explored. This provides the possibility to resolve spatial asymmetry in the release of different neurotransmitters across a single cell. Furthermore, a comparison is made between amperometry and FSCV for electrochemical imaging. It is demonstrated that the lower temporal resolution in FSCV compared to amperometry is the result of the slower rate of sampling in combination with the non-consumptive nature of FSCV resulting in a longer contact time between the electrode and the released molecule. The fact that the neurotransmitter is present in the gap between the cell and the electrode for a long time leads to significant cross talk between the individual electrodes of the array, a phenomenon that is not as pronounced when using amperometric detection.

In paper II, the amperometric response from exocytotic release at PC12 cells is compared using ring-shaped versus disk-shaped electrodes. The difference in diffusional distance, based on the geometries of the two electrode types, is explored to evaluate the rate of diffusion of dopamine in the extracellular matrix (ECM) surrounding the cell. The data indicate that the diffusion coefficient of dopamine in the ECM is almost an order of magnitude smaller than that in free solution. This is hypothesized to be the result of interactions between the neurotransmitter and the constituents of the ECM.

In paper III, exocytosis from PC12 cells is studied with the intent to discover if a post-spike transient occurs from transmitter released during the vesicle closing at the end of exocytosis. Amperometric peaks corresponding to release from these cells frequently display a slight increase in current immediately preceding the event. This feature is called the pre-spike foot and it stems from the efflux of neurotransmitter through a nanometric fusion pore formed as an initial stage of the fusion process. In this paper a stable plateau current has indeed also been identified during the decay part of the peak. I term this feature the post-spike foot, and hypothesize that it is the result of a formation of a nanometric fusion pore as the vesicle closes up again following neurotransmitter release. This hypothesis is

strengthened by the fact that the flux of molecules though the closing pore is smaller than the flux through the opening pore, as demonstrated by a smaller post-spike foot than pre-spike foot current. This indicates that the concentration of neurotransmitter inside the vesicle is lower during vesicle closing than during its opening, a natural consequence of the preceding release of content. Furthermore, the decrease in foot current can be tuned by incubating the cells with high curvature phospholipids, a treatment that is believed to alter the fraction of neurotransmitter released during exocytosis.

In paper IV the dimensions of lipid nanotubes formed from membranes of various lipid compositions have been examined. This work builds on a previous publication where Fick's first law of diffusion was used to measure the diameter of lipid nanotubes formed from giant unilamellar vesicles constructed from soybean lipids. In paper IV the influence of membrane lipid composition on nanotube diameter has been studied by altering the lipids in the initial preparation, or alternatively by incubating the vesicles with lipids after they are formed. By adding the cone shaped lipid phosphatidylethanolamine (PE) the diameter of the nanotube is decreased in a concentration dependent manner. The data also suggest that the formation of the nanotube causes segregation of lipids in the membrane, where the highly negatively curved lipid PE is believed to accumulate in the inner leaflet of the nanotube.

In paper V the artificial cell model is used to study the membrane dynamics of fusion pore expansion. The model is brought one step closer to the conditions in a living cell by the exploration of plasma membrane vesicles from PC12 cells as the artificial cell and vesicle membrane. When studying exocytotic release in this model I made an exciting observation; this membrane releases molecules through two distinct mechanisms; full and partial distension. The two modes of release show distinctively different kinetics, making them easy to distinguish with amperometric detection. It appears that the partial distension mode may be parallel to the extended kiss-and-run mechanism of release believed to occur in cells, making this an exciting new model for studying transient fusion and fractional release of neurotransmitter.

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