THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Microanalytical Approaches to Probe Exocytosis and Vesicle Content at Cells and Artificial Cells

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2016

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ISBN: 978-91-7597-455-2
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Doktorsavhandlingar vid Chalmers tekniska högskola
Ny serie nr 4136
ISSN: 0346-718X

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Cover Picture: illustration of the exocytosis process in the middle and four different approaches to study the exocytosis process including amperometry, fluorescence microscopy, artificial cell and vesicle electrochemical cytometry.

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Abstract

Better understanding of neuronal communication is important to understand the general functioning of the brain and get more knowledge about the underlying reasons of neurodegenerative diseases like Parkinson´s disease. Neurons communicate through electrical and chemical signaling. In chemical signaling through a process called exocytosis a vesicle filled with neurotransmitters fuses into the cell membrane and releases its content. There are many different modes of exocytosis but it can be mainly divided into three modes including full release, kiss-and-run, and extended kiss-and-run modes.

While a lot of proteins involved in exocytosis have been well characterized, the critical role of membrane lipids has not been fully understood. Therefore there is an increasing appreciation of the importance of the lipids to various aspects of exocytosis. In the first three papers of this thesis I have tried to focus on the role of lipids on the formation and dynamic of fusion pore using artificial cell models. In paper I the effect of different membrane lipid compositions on the dimension of lipid nanotube is studied and it has been shown that high curvature lipids are sorted into the high curvature region of the lipid nanotube where they facilitate tube membrane curvature. In paper II an artificial cell model has been developed that can mimic two modes of exocytosis: full and partial release. It has been shown in this study that the determining factor to switch between modes is membrane tension. This developed model further was used to study the effect of cholesterol on exocytosis in paper III. In this paper I have clearly shown that the cholesterol dependent biophysical properties of the membrane directly affect the exocytosis kinetics and that membrane tension along with membrane rigidity can influence the fusion pore dynamics and stabilization.

As one of the biggest questions is what percentage of vesicle content is released during exocytosis and what factors affect the released amount, developing a technique to quantify the content of vesicles is highly appreciated. In paper IV I present a technique called vesicle electrochemical cytometry where we can quantify the vesicular content of chromaffin cells vesicles. In this system isolated chromaffin vesicles adsorbed at the polarized electrode surface and release their content with time. In paper V the mechanism of mammalian vesicle rupture at the electrode surface is investigated. It appears that following adsorption to the surface of the polarized electrode, electroporation leads to formation of a pore at the interface between a vesicle and the electrode. Using a bottom up approach, lipid-only transmitter-loaded liposomes were used to mimic native vesicles and the rupture events occurred much faster in comparison with native vesicles. In paper VI I have confirmed that by manipulating membrane properties with excited fluorophore we can make the membrane more vulnerable to electroporation and increase the fusibility of vesicles.

Keywords: *Exocytosis, amperometry, artificial cell models, vesicle electrochemical cytometry, fusion pore*

List of Publications

This thesis is based on the following publications and manuscripts, referred to in the text by Roman numerals:

I. "Composition-based strategies for controlling radii in lipid nanotubes".
M.E. Kurczy, L.J. Mellander, **Neda Najafinobar**, and A-S Cans. *PLoS One*, 2014, 9 (1): 81293.

II. "Observation of two modes of exocytosis in an artificial cell model".
L.J. Mellander, M.E. Kurczy, **Neda Najafinobar**, J. Dunevall, A.G. Ewing and A-S Cans. *Nature Scientific Reports,* 2014, 4: 3847

III. "Cholesterol alters the dynamics of release in protein independent cell models for exocytosis".
Neda Najafinobar, L. J. Mellander, M. E. Kurczy, J. Dunevall, T. B. Angerer, J. S. Fletcher and A-S. Cans. Submitted.

IV. "Characterizing the catecholamine content of single mammalian vesicles by collision-adsorption events at an electrode".
J.Dunevall, H.Fathali, **Neda Najafinobar**, J.Lovric, J.Wigstrom, A-S.Cans, A.Ewing. *J Am Chem Soc,* 2015, 137 (13): 4344-6.

V. "On the mechanism of electrochemical vesicle cytometry: chromaffin cell vesicles and liposomes".
Jelena Lovrić,* **Neda Najafinobar**,*Johan Dunevall,Soodabeh Majdi, Irina Svir, Alexander Oleinick, Christian Amatore, Andrew G. Ewing. *Faraday Discussions*, 2016, DOI: 10.1039/C6FD00102E

VI. "Excited fluorophores enhance the opening of vesicles at electrode surfaces in vesicle electrochemical cytometry".
Neda Najafinobar, Jelena Lovric, Soodabeh Majdi, Johan Dunevall, Ann Sofie Cans, Andrew Ewing. Submitted.

* These authors contributed equally to this work

Related Papers not Included in the Thesis

VII. "Lithographic Microfabrication of a 16-Electrode Array on a Probe Tip for High Spatial Resolution Electrochemical Localization of Exocytosis". J. Wigström, J. Dunevall, **N. Najafinobar**, J. Lovric, J. Wang, A.G. Ewing and A-S Cans. *Analytical chemistry,* 2016, 88 (4): 2080-2087.

VII. "Study the Effect of Dimethyl sulfoxide (DMSO) on Vesicular Content Release in Single Adrenal Chromaffin Cells with Nano-tip Conical Carbon Fiber Microelectrodes". Soodabeh Majdi, **Neda Najafinobar**, Jelena Lovric, Johan Dunevall, Andrew Ewing. Submitted.

IX. "Measurement of exocytotic release, from a population of PC12 cells, by amperometry and QCM-D".J.Wigström, **N.Najafinobar,** A-S. Cans. Manuscript in preparation.

X. "Applying Intracellular Cytometry to Study the Effect of ATP on Exocytosis using Chromaffin Cells". Soodabeh Majdi, **Neda Najafinobar,** Jelena Lovric, Johan Dunevall, Andrew Ewing. Manuscript in preparation.

CONTRIBUTION REPORT

Paper I. Performed part of the experiments and wrote part of the manuscript.

Paper II. Performed all experiments together with Lisa Mellander and was involved in reading and revising the paper.

Paper III. Planned and performed all experiments and wrote the paper.

Paper IV. Performed part of the experiments and was involved in reading and revising the paper.

Paper V. Planned and performed all experiments together with Jelena Lovric and was involved in writing, reading and revising the paper.

Paper VI. Planned and performed all experiments and wrote the paper.

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List of Abbreviations

Central nervous system (CNS)
Peripheral nervous system (PNS)
Parkinson's disease (PD)
Alzheimer's disease (AD)
Amyotrophic lateral sclerosis (ALS)
Acetylcholine (Ach)
Norepinephrine (NE)
Dopamine (DA)
Serotonin (5-HT)
Gamma aminobutyric acid (GABA)
Adenosine triphosphate (ATP)
Guanosine-5'-triphosphate (GTP),
L-dihydroxyphenylalanine (L-DOPA)
Dopamine transporter (DAT)
Noradrenaline (NA)
Phenylethanolamine N-methyltransferase (PNMT)
Phosphatidylcholine (PC)
Phoshphatylethanolamine (PE)
Phoshphatidylserine (PS)
Phoshphatidylinositol (PI)
Large dense core vesicles (LDCV)
Vesicle associated membrane protein (VAMP)
Small synaptic vesicles (SSVs)
Clathrin-mediated endocytosis (CME)
Phosphatidic acid (PA)
Lysophosphatidylcholines (LPC)
Total internal reflection fluorescence microscopy (TIRFM)
Stimulated emission depletion microscopy (STED)
Transmission electron microscopy (TEM)
Enhanced green fluorescent protein (EGFP)
Fast scan cyclic voltammetry (FSCV)
Using microelectrode arrays (MEAs)
Capillary electrophoresis (CE)
Pheochromocytoma cells (PC12)
Giant unilamellar liposome (GUV)

To my beloved brother
Amir

Chapter 1

The Nervous System

The nervous system consists of two main parts: a central nervous system (CNS) that includes the brain and spinal cord and a peripheral nervous system (PNS) that mainly consists of neurons. Neurons in the PNS are divided into three categories: motor neurons that are responsible for voluntary movements, autonomous neurons that mediate involuntary functions, and the enteric nervous system that controls the gastrointestinal system. The spinal cord is the main pathway for information, which connects the brain in the CNS with neurons in the PNS and serves to transfer messages into any part of the body.

Glial cells are another cell type that makes up the bulk of the nervous system. Glia and neurons have many structural and molecular characteristics in common since they both develop from common neuroepithelial cells of the embryonic nervous system. Glial cells have two main categories: macroglia and microglia. Macroglia can further be divided into oligodendroglia that are responsible for making myelin sheets around the axon in the CNS, ependyma cells that make myelin sheets in the PNS, and astrocytes that are involved in many functions such as supporting neuronal structure and holding them in place, providing food for the neurons by releasing growth factors, and improving the efficiency of signaling between the neurons by taking up neurotransmitters and K^+ ions from the extracellular space. Microglia on the other hand are responsible for the destruction of pathogens and to remove dead neurons.[1] Glial cells have an important role in a well functioning nervous system and changes in their function can contribute to neurodegenerative diseases. Examples of these changes can be gliosis of astrocytes or the activation of microglia. It has been shown that these reactive and inflammatory responses play an important role in damaging the neuron in many neurodegenerative diseases including Parkinson’s disease (PD), Alzheimer’s disease (AD) and Amyotrophic lateral sclerosis (ALS).[2]

Neurons

It has been estimated that the human brain has about 85 billion neurons and 10 times more glial cells.[3] The function of these neurons is to process and transmit information from the brain to other parts of the body including other nerve cells, gland cells, and muscles. Neurons are different from other cell types in the body in the sense that they are polarized and have different structural cell regions including: cell body, dendrites,

and an axon, each with a specific function (Fig. 1).[4] The cell body or soma isolates different organelles that are crucial for cell functioning such as the nucleus or mitochondria from the extracellular fluid. The dendrites and axon extend from the cell body and usually divide into smaller branches. The function of axons and dendrites is generally to transfer and receive information from the adjacent neuron, respectively. Axons can send signals over a distance of up to 2 meters with speeds of 1 to 100 meters per second. In the classical model, the signal is transferred from the soma to axon terminals or so called synapses where it is transmitted to another neuron either electrically or chemically.

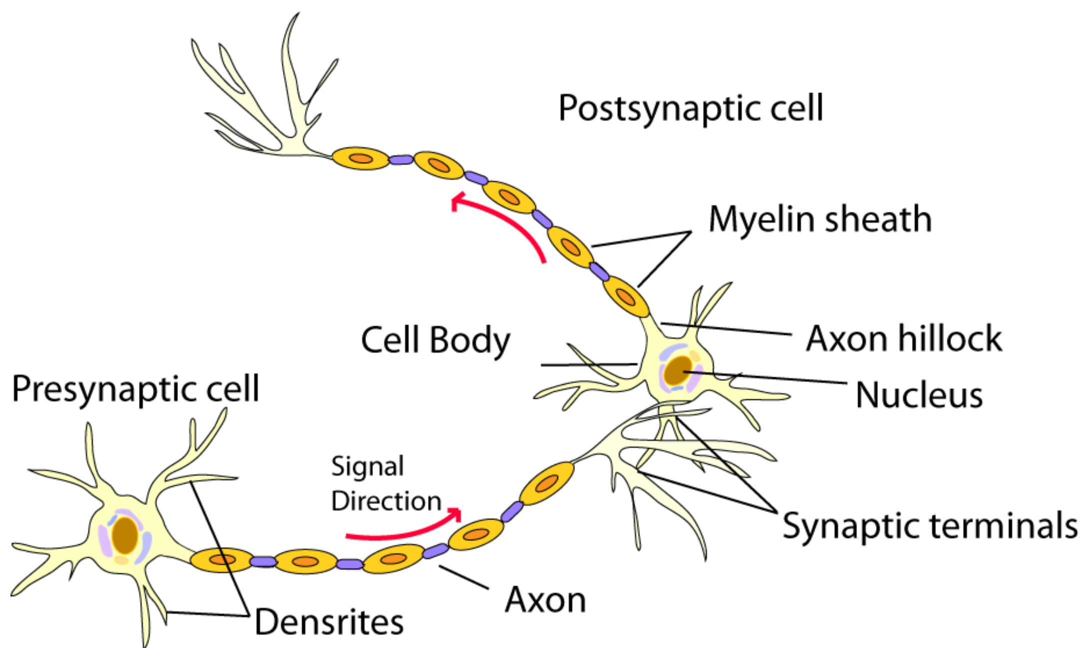

Figure 1. Illustration of neuronal structure. Neurons have three distinct parts including the cell body or soma, the axon and dendrites. In most neurons the signal is transferred from the axon terminal of the presynaptic cell to the dendrites of the postsynaptic cell where the release of neurotransmitters occurs.

There are many different ways to categorize neurons considering their morphology and function. One general way to classify neurons is based on their polarity (Fig. 2). Neurons can be unipolar where the axon and dendrites extend from the same place on the cell body, bipolar where the axon and dendrites grow on opposite sides of the soma and multipolar when neurons have single axon with multiple dendrites that are separate from the axon and make it possible to receive a lot of information from other neurons through multiple connection points.[1]

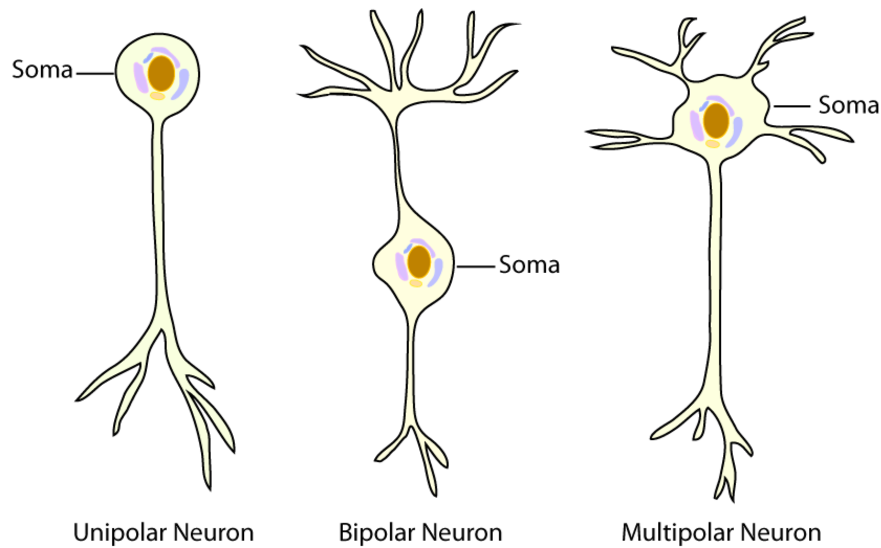

Figure 2. Neurons can be divided into unipolar, bipolar and multipolar types based on their polarity.

As mentioned earlier, neurons also have polarized potential across their membrane with a negative potential of about -60 up to -80 mV inside the cell with respect to the extracellular fluid. This negative potential is called the resting potential and is caused by asymmetric distribution of ions across the cell membrane. The concentration of Na^+ and Cl^- ions is high outside of the cell, whereas the concentration of K^+ ions and organic anions is high inside the cell and the high selectivity of ion channels and a sodium/potassium pump maintain this ion distribution. During the resting potential, ion channels on the cell membrane are not permeable to Na^+ ions while they are more permeable to K^+ ions. There are also sodium-potassium pumps in the membrane that pump Na^+ ions out and K^+ ions inside the cell. The difference in concentration that is built up by the pumps, and maintained by ion channels, results in an electrochemical potential across the membrane with it being negative inside the cell versus the outside.[1]

When one neuron sends signal to adjacent neuron, it undergoes a very rapid depolarization to about +40 mV in just 0.5 milliseconds. This rapid depolarization results from the opening of Na^+ channels on the membrane that makes it possible for Na^+ ions to pass across the cell membrane toward the inside of the cell at extremely rapid rates and provides a large flow of local electrical charge. In neurons, up to 100 million ions can pass through a single channel in one second.[5] The positive potential inside the cell is called an action potential and is mostly initiated in the axon hillock where the density of sodium channels is high. Once an action potential is initiated in one region of a neuron such as axon hillock, the neighboring voltage-gated channels are opened allowing it to propagate along the axon and invade the synapses where it can initiate the process of synaptic transmission. Since the nerve cell membrane is thin and surrounded by two conducting media, it has a high capacitance which results

in poor conduction of the action potential along the axon. To allow better conduction of the action potential the axon is myelinated with glia cell. The myelin sheath is a layer of fatty substance that wraps around the axon and produces electrical insulation. Therefore it reduces the membrane capacitance and increases the membrane resistance thus allowing rapid movement of the action potential.[1]

Neuronal Communication

The communication between neurons is thought to generally occur through a process known as synaptic transmission where the signal is transferred from the axon terminal of a presynaptic cell to the dendrites of a postsynaptic cell. All synapses have a zone of opposition and based on the structure of the opposition, synapses can be either electrical or chemical.[4] An electrical synapse occurs when the presynaptic terminal and postsynaptic cell are in very close apposition (around 4 nm) at a region known as a gap junction. In this less general case, the cytoplasm of the presynaptic and postsynaptic cells is physically connected through specialized protein structures called gap junction channels. These protein channels make it possible for the current generated by an action potential in the presynaptic neuron to directly enter the postsynaptic cell. Therefore, electrical signaling compare to chemical signaling is faster and less complex, but chemical synapses allow more variation in signal type and both excitatory and inhibitory signals can occur.

At chemical synapses, neurons are not directly connected and are separated by a gap known as the synaptic cleft that is approximately 40 nm. Therefore, to transfer the signal to postsynaptic cell, the presynaptic cell needs to release signaling molecules or so called neurotransmitters. In chemical signaling, the release of neurotransmitters is triggered when the action potential reaches the axon terminal and initiates the opening of Ca^{2+} channels. Ca^{2+} ions enter the cell starting a cascade of reactions to initiate neurotransmitter release via the process of exocytosis. This is a critical process and is described in more detail in the next section. Neurotransmitters released at the axon terminal diffuse across the synaptic cleft to the adjacent neuron, and bind to specific receptors and regulate the opening and closing of ions on the membrane of the target cell. A diagram illustrating these processes is shown in Figure 3. Each neurotransmitter binds to specific receptors, for example the dopamine receptors bind to the neurotransmitter dopamine and have little affinity for other neurotransmitters such as serotonin. Therefore the factors that determine the resulting signal in any given scenario are the properties of the receptor that the transmitter binds to and not the identity of the transmitter itself. Thus, several different effects can be produced by a single transmitter depending on the different types of receptors that it activates and this results in a wide variety of synaptic actions.

Depending on which receptors the neurotransmitter binds to, the resulting effect can be excitatory, inhibitory or modulatory. Excitatory and inhibitory signals facilitate and inhibit an action potential in the postsynaptic neuron, respectively. If the response is excitatory the potential inside of the target cell becomes less negative and causes the cell to depolarize, whereas an inhibitory response causes the potential of the cell to become more negative and results in hyperpolarization of the postsynaptic cell. There are several means to depolarization or hyperpolarization. For example, depolarization can be caused by opening the ligand gated ion channels that lead to the flow of Na^+ ions inside the cell. In contrast an example of hyperpolarization can occur through opening of Cl^- channels and the subsequent flow of Cl^- ions inside the cell or opening of K^+ channels leading to movement of K^+ ions to the outside of the cell. After neurotransmitters have transferred the signal, these molecules detach from the receptors and diffuse back into the synaptic space. The synaptic space is then cleaned of these neurotransmitters by several different mechanisms. For instance, they can undergo enzymatic degradation in the synaptic cleft or be taken back up into the presynaptic axon terminal by active transport through a transporter or reuptake pump.[1] Neurotransmitters can also diffuse into the extracellular solution to be used elsewhere or to be degraded.

Although it is believed that neurons mostly communicate through synapses, there is another mode of communication in which released transmitters deliberately move out of the synaptic cleft to signal a more distant target. This is called volume transmission and the main purpose of volume transmission appears to be neuromodulation.[6] Neuromodulators are neurotransmitters that are not taken up by the transporters on presynaptic cell or broken down into metabolites and therefore stay in the cerebrospinal fluid for longer time and modulate signals at a longer distance.[1, 7, 8] Neuromodulation is an important property of an individual neuron because it helps the nervous system to regulate its function to an environment that is continuously changing. It has also been shown that neuromodulation is the basis for many long lasting changes in animal behavior.[9]

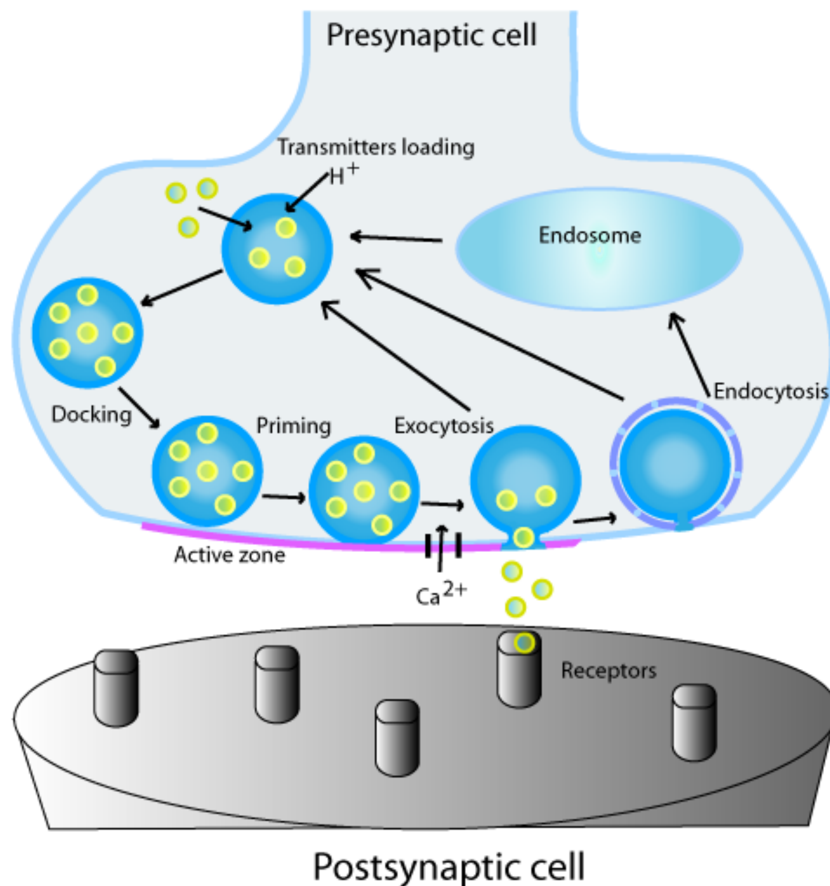

Figure 3. This figure illustrates an axon terminal in a presynaptic cell. The vesicle filled with neurotransmitters docks into the cell membrane and through a process called exocytosis it fuses into the cell membrane and releases its content.

Neurotransmitters

It is hard to give an accurate definition of a neurotransmitter since with time new types of molecules have been recognized as neurotransmitters. In general, molecules used to transfer messages from the presynaptic neuron to postsynaptic neuron are called neurotransmitters. Most functions of the body are mediated through neurotransmitters including emotional response and movement. Neurotransmitters can be divided into different categories based on their structure or function. One general way is to divide them into two main groups including small molecules and neuroactive peptides.[1] Small neurotransmitters based on their chemical structure can be further divided into: 1) acetylcholine (Ach); 2) monoamines including norepinephrine (NE), dopamine (DA), serotonin (5-HT); 3) amino acids such as glutamate, gamma aminobutyric acid (GABA), aspartate, histamine; and 4) purines such as adenosine, adenosine triphosphate (ATP), guanosine-5'-triphosphate (GTP), and their derivatives. Neurotransmitters in this group are usually packed in small

synaptic vesicles with a diameter around 40 to 60 nm and can be involved both in synaptic and volume transmission. For instance, GABA, the main inhibitory transmitter, or glutamate, the main excitatory neurotransmitter, release via synaptic transmission, whereas dopamine aside from synaptic transmission can also communicate through volume transmission.[7, 10, 11] In contrary to small neurotransmitters, neuroactive peptides are thought to be packed in large dense core vesicles and are used as modulators for volume transmission.[12, 13] Different neurotransmitters have different roles in the body and high or low levels of neurotransmitters can cause neurodegenerative diseases. For example, Schizophrenia is a mental illness that is associated with a high level of dopamine, whereas the symptoms of Parkinson's, another degenerative disease, are caused by a low level of dopaminergic neurons.[14]

Dopamine

Dopamine, perhaps the most studied neurotransmitter, was first recognized as neurotransmitter in 1958 by Arvid Carlsson and his co-workers where they showed dopamine is more than a precursor for norepinephrine and epinephrine and it is a neurotransmitter itself.[15] Since then many studies have been devoted to better understand the role of dopamine in the brain and body and it has been shown that dopamine is involved in the reward system[16] and also in the control of movement and the decrease in dopamine levels in the striatum leads to difficulties in movement that are the hallmark of Parkinson´s disease.[17]

Tyrosine, an essential amino acid, is the precursor for dopamine biosynthesis. In a common biosynthetic pathway tyrosine is converted to L-dihydroxyphenylalanine (L-DOPA) via the enzyme tyrosine hydroxylase and then L-DOPA is converted into dopamine via the enzyme aromatic L-amino acid decarboxylase. Dopamine is then packed into synaptic vesicles and is released into extracellular fluid where it binds to specific receptors. There are at least five subtypes of dopamine receptors: D1 to D5 and all of them function as metabotropic, G protein-coupled receptors, where they transfer the message through a second messenger system. As it has been mentioned earlier dopamine can also function as neuromodulator where its signaling happens through volume transmission and it binds to the receptors of hundred postsynaptic cells over a large area.[7, 11, 18] The effect of dopamine on the postsynaptic cell can be either excitatory or inhibitory depending on the receptor it binds to. For example D2-like receptors composed of D2, D3, D4 are mostly inhibitory and function ultimately through the activation of K^+ channels. After synaptic transmission, the excess amount of dopamine is either broken down into metabolites such as homovanillic acid using different enzymes or is transported back into the cell by dopamine transporters (DAT).

Norepinephrine

Norepinephrine (NE), also called noradrenaline (NA), is synthesized from dopamine using dopamine beta-hydroxylase. Norepinephrine synthesis occurs predominantly inside the vesicle of noradrenergic neurons while the synthesis of dopamine occurs in the cytoplasm. Similar to dopamine, norepinephrine can also act as a neuromodulator[7, 18] as well as a transmitter and all receptors specific for norepinephrine function as G-coupled protein receptors. There are two main categories of norepinephrine receptors known as alpha and beta.[19] There is much evidence showing the existence of a sub population of alpha and beta receptors in the periphery with different sensitivity to various agonists. Alpha receptors are composed of alpha 1 and alpha 2 and beta receptors are composed of beta 1, beta 2, and beta 3 sub types. Alpha 1 and all beta receptors are mostly excitatory receptors while alpha 2 is usually inhibitory. It has been shown that one sub type of alpha receptors that is located on the presynaptic membrane of noradrenergic nerve endings is responsible for modulation of the efflux of NE from the nerve terminal. It has been observed in this study that stimulation of these receptors decreases the release of NE, whereas blocking them with alpha blockers increases the release. Regulating the release of NE from cerebral cortical neurons also appears to be via similar receptors.[20]

Epinephrine

Epinephrine is synthesized by methylation of norepinephrine via the enzyme phenylethanolamine N-methyltransferase (PNMT). In contrast to norepinephrine, which is synthesized inside granules, for epinephrine synthesis from norepinephrine, norepinephrine has to leave the granules in order to be N-methylated in the cytoplasm and then is taken up and stored in the granules again.[21] The receptors for epinephrine are the same as norepinephrine. However it has been shown that norepinephrine has a more potent effect on beta 1 receptors, whereas epinephrine has a more potent effect on beta 2 receptors. Several studies have been carried out to determine the factors affecting the affinity of different receptors to epinephrine, but the original low physiological affinity of epinephrine to beta 1 receptors has made these studies challenging. Even for beta 2 receptors, where the affinity for epinephrine is high, some experimental manipulations are required in order to bring the epinephrine affinity at beta 2 into a measurable range.[22]

Exocytosis

Chemical synaptic transmission can be divided into 5 steps: 1) synthesis of neurotransmitters and storage of them inside vesicles, 2) transportation of vesicles to synapses, 3) release of transmitters, 4) interaction of the neurotransmitters with receptors at the postsynaptic membrane, and 5) removal of the transmitters from the synaptic cleft. Because the axon and terminals often are at great distance from the cell body, effective transport mechanisms are crucial for sustaining the function of synapses and passive diffusion is too slow to deliver vesicles over this distance. Vesicles move toward axonal terminals by fast axonal transport, which uses kinesin and a variety of kinesin related proteins as motor molecules. These proteins use ATP as a source of energy to transport vesicles to the axon terminal. Once vesicles get to the axonal terminal they release their content through a process called exocytosis. This is described in some detail below.

Vesicles (Small Synaptic Vesicles, Large Dense Core Vesicles)

Neuronal communication relies on neurotransmitter loaded synaptic vesicles that release their content at synapses. In 1954 Bennett and co-workers showed that there were a large number of synaptic vesicles at the synaptic regions encountered in sections of frog sympathetic ganglia and earthworm nerve cord neuropile.[23] Synaptic vesicles have a lipid composition of 40% phosphatidylcholine (PC), 32% phoshphatylethanolamine (PE), 12% phoshphatidylserine (PS), 5% phoshphatidylinositol (PI) and 10% cholesterol.[24] However, in another study done by Jahn and coworkers the cholesterol percentage was higher at around 40%.[25] The vesicles membranes contain V-type ATPases that pump protons inside the vesicle and generate low pH (around 5.5) inside the vesicle. They also contain proton-coupled neurotransmitter antiporters to uptake neurotransmitters from the cytosol into the vesicle while letting protons out of the vesicle. Low molecular weight neurotransmitters such as acetylcholine, dopamine, norepinephrine, epinephrine and many others are encapsulated inside small synaptic vesicles ranging from 40 to 60 nm in diameter. Neuropeptides are packaged into large dense core vesicles (LDCVs) that range from 90 to 250 nm in diameter. LDCVs are defined by the presence of a dense core that is composed mostly of a densely packed matrix of semi-crystalline monoamine and acidic proteins known as chromogranins. The dense core is surrounded by a solution of solubilized monoamine that is called the halo. It has been shown in several studies that the transmitter levels inside the vesicle can be manipulated using different pharmaceutical drugs such as L-DOPA (a biosynthetic precursor to intracellular dopamine) and reserpine (an inhibitor of the vesicular monoamine transporter that is responsible to transfer dopamine into the vesicle). These drugs have been shown to directly affect the size of the vesicle.[26] Treating

vesicles with L-DOPA increases the size of the halo while reserpine decreases the size of the vesicular halo.[27, 28]

Vesicles Release Through Docking, Priming and Fusion

Each neuron contains millions of vesicles that are filled with neurotransmitters. When one neuron communicates a signal to an adjacent neuron the vesicle in the presynaptic cell is transported close and docked to the plasma membrane of the cell with the help of complexes formed between t-SNARE proteins on the cell membrane and v-SNARE proteins on vesicle membrane. When two structurally stable bilayers such as the synaptic vesicle and plasma membrane want to fuse together they have to overcome a large unfavorable activation energy and this is accomplished by the SNARE protein complex.[29]

SNARE proteins are all identified by their simple domain structure known as the SNARE motif that consists of 60-70 amino acids that are organized in heptad repeats. These amino acids can assemble into reversible four helix bundles.[30] The docking of a vesicle at the cell membrane is believed to occur through the binding of vesicle SNARE proteins named synaptobrevin, which counts as vesicle associated membrane protein (VAMP) and plasma membrane SNARE proteins that include syntaxin and SNAP-25 (synaptosome-associated protein). The four-alpha-helix bundle of SNARE complex is made up by one alpha-helix of syntaxin, one alpha-helix of synaptobrevin and two alpha-helix of SNAP-25. The combination of these three proteins is extraordinarily stable and the energy released here from this combination brings the two negatively charged membranes of the vesicle and cell together. This entire process is initiated by calcium influx into the cell following an action potential.

The action potential, beginning at the axon hillock, travels along the axon to the synaptic terminal where this results in opening of voltage-gated calcium channels, which are the trigger for transmitters release. Several studies have shown that the amount of transmitters released is dependent on the extracellular Ca^{2+} concentration and an increase in Ca^{2+} concentration enhances transmitter release, while lowering the concentration decreases synaptic transmission.[1, 31, 32] Since the release of neurotransmitters is an intracellular process, these findings show that Ca^{2+} ions must enter the cell to affect the release process.[32] It has been shown that calcium channels are more abundant in the active zones of presynaptic terminals.[33] Ca^{2+} ions flowing inside the cell cause docked vesicles to fuse with the cell membrane and release their content. After Ca^{2+} ions enter the cell they are rapidly buffered by calcium-binding proteins and therefore cannot diffuse long distances from the site of the entry.

The regions in neurons called active zones are the points where small synaptic vesicles dock and participate in synaptic secretion through the exocytosis process. These regions have high local SNARE concentration that has been shown is essential for efficient fusion.[30] The number of small synaptic vesicles found in an active zone is dependent on the brain region and synapse type.[34] Postsynaptic receptors are located close to the active zones on presynaptic cells and therefore allow rapid and efficient secretion in the synaptic cleft.[35, 36]

Over time the idea of different types of vesicles with distinct characteristics for release has been strengthened.[37] These are divided into subgroups. The first group is the readily releasable pool (RRP) that contains vesicles that are already docked to the presynaptic active zones and primed for release. It has been shown that these vesicles are depleted rapidly upon high frequency electrical stimulation or hypertonic shock in hippocampal boutons.[38, 39, 40] The second group, known as the recycling pool, contains about 5 to 10% of all vesicles and these are released upon moderate stimulation. The reserve pool is the third group of vesicles that contains around 80 to 90% of all vesicles and these are only released upon strong stimulation. It has been shown in different studies that vesicles in the reserve pool only release at hippocampal boutons when prolonged high potassium stimulation is applied.[38]

LDCVs, in contrast to small synaptic vesicles (SSVs), are not localized in active zones and they usually do not release at synapses.[38]

Different Modes of Exocytosis

It is believed that when a vesicle and the cell plasma membrane come in close contact with each other and fuse, a small lipid nanotube or fusion pore forms that then expands to various extents determined by the mode of exocytosis release. There are many different modes of exocytosis[41] but it can be mainly divided into three modes including full release, kiss-and-run, and open and closed mode (Fig. 4). Full release occurs when the vesicle distends completely with the cell membrane and releases all its content. For a long time this mode of release was considered to be the main mode for neuronal communication.[42, 43] However, recently there has been a wealth of evidence showing that fractional release is the most dominant mode of exocytosis in chromaffin and PC12 cells as well as neuronal cells.[43, 44, 45] In the open and closed mode the fusion pore is formed and then opens to allow more release of its content before the pore closes and re-seals.[46] The kiss and run mode of release occurs when the pore is formed and close again without further opening.[47] Also there is a mode called flickering and this mode releases very small quantities of transmitters.[48] There are many studies showing that changing different parameters such as the frequency of stimulation[49] or temperature[50] can alter the released amount and mode of exocytosis

and therefore, understanding what determines the mode and amount of release is crucial.

Different studies applying different approaches have provided evidence for fractional release in neuronal communication. A study carried out on PC12 cells using electrochemical cytometry is perhaps the key work to show that fractional release is the dominant mode of release and only 40% of vesicular content is released during exocytosis.[51] Quantifying the vesicular content of PC12 cells by applying the recently developed technique known as intracellular vesicle electrochemical cytometry has also confirmed that vesicles do not dilate all the way resulting in partial release of the vesicular contents and only 64% of vesicular content is then released.[52] Several other studies also have shown that by manipulating the cell through changing the lipid composition of the cell membrane[46, 53] or the osmolality of the solution around the cell,[54, 55] the amount that is released can be increased or decreased confirming that not all the vesicular content is released during exocytosis. There are also studies done on neuronal cells that confirm the fractional release. For instance the study done on hippocampal dopamine neurons showed that only around 30% of vesicular content is released through the fusion pore.[48]

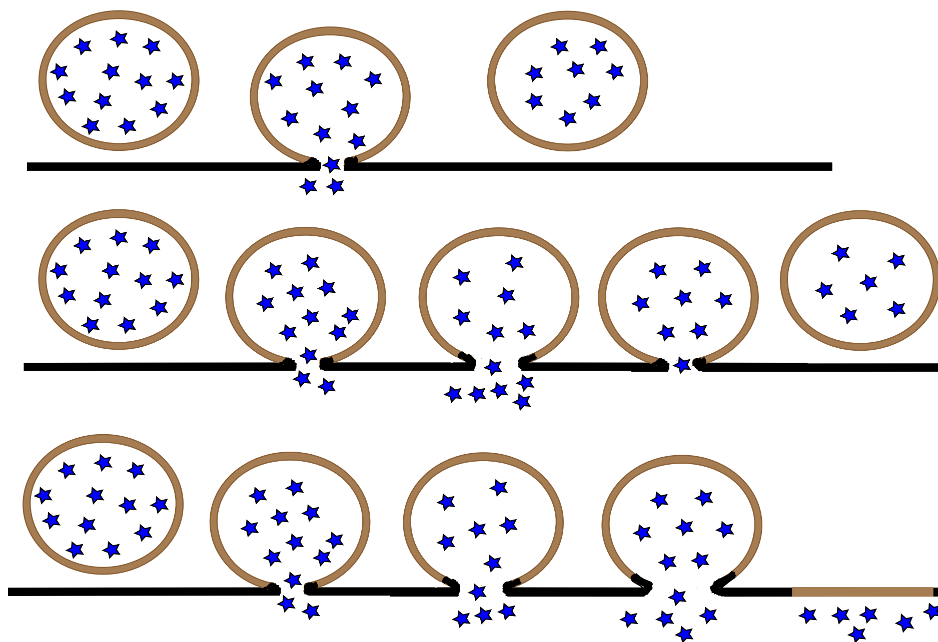

Figure 4. Three different exocytosis modes have been shown including full release, open and closed, and kiss-and-run mode, respectively from the bottom.

Endocytosis

After vesicle fusion and full distention, there is a need to recapture the vesicular membrane. In this case excess membrane material in the cell membrane, resulting from incorporation of vesicle membrane into the cell plasma membrane, is retrieved by a process called endocytosis.[56, 57] Endocytosis can occur through different pathways but the most well studies pathway is clathrin-mediated endocytosis (CME). In this process clathrin proteins cover the fused vesicle membrane and form structures called clathrin coated pits and then pinch off the vesicle from the plasma membrane back into the cytoplasm. This is followed by removal of clathrin from the retrieved vesicle membrane and this vesicle later becomes a new synaptic vesicle and joins the secretory vesicle pool.[57]

In the case of kiss-and-run and open and closed exocytosis, release of neurotransmitters occurs through the fusion pore opening and is not followed by complete distension of vesicle into the cell membrane. After partial release of vesicle content the fusion pore closes and the vesicle is retrieved back through fast endocytosis.

Chapter 2

The Role of Membrane Lipids in Exocytosis

In general, lipids are very important for cell function and are used for different purposes such as energy sources, enzymatic cofactors, hormones and intracellular signaling molecules. However the most important role of lipids in cell is that they make the main structural component of the biological membrane. The cell membrane mainly consists of phospholipid bilayer that is a thin oily film with 5 nm thickness. This oily film has soft and flexible structure which makes it possible for the cell to go through different shape transitions including budding, fission and fusion that are necessary for cellular function such as cell division or exo- and endocytosis. The membrane bilayer also has a crucial role in separating the cellular content from the surrounding environment and regulates the trafficking of different molecules in and out of the cell by several different types of protein channels and pumps that are embedded in the cell membrane. The membrane bilayer consists mainly of phophatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylserin (PS), phosphatidylinositol (PI), and phosphatidic acid (PA).[1]

Lipids

Lipids are divided into different categories based on their functional structure. The major group of lipids that is also the main component of the cell membrane is phospholipids. Phospholipids consist of distinct sections: a hydrophilic head group and a hydrophobic hydrocarbon tail group and a backbone connecting the tail to the head group. Each of these phospholipids has their own specific structure in terms of the length of hydrocarbon tail, the number of unsaturated bonds, and the chemical composition of the head group that can give different properties to the lipid. For instance, based on the composition of the head group, phosphatidylserine (PS) is negatively charged at neutral pH while phosphatidylcholine (PC) is neutral and therefore depending on the lipid composition of the membrane the overall charge of the membrane can be different. Different structures of lipids can also influence the biophysical properties of the cell membrane such as curvature and rigidity and this is explained in more detail in the following section.

Sphingolipids constitute another category of lipids with their nonpolar backbone being ceramide. This type of lipid has saturated tail groups that make it possible for them to pack more tightly and form a solid gel phase. Steroids are also another

category of lipids that are composed of a fused ring system connected to a non-polar hydrocarbon tail and a polar head group. Cholesterol is perhaps the most important lipid in this group with a large hydrophobic structure and a OH-group as polar head group.[1]

Bilayer Properties are Dependent on Lipid Composition

In aqueous environments phospholipids make bilayers rapidly because the polar head groups face the water, and the nonpolar tail groups face each other.[58] As mentioned earlier, different lipids with different geometries and different chemicals as head groups can give different properties to the bilayer. Lipids with longer hydrocarbon tails have more hydrophobic interaction with the tail groups of adjacent lipids and therefore they become more tightly packed.[59] The packing density of lipids in a chain is also related to the number of unsaturated bonds in the hydrocarbon tail and also the size and chemical composition of the head group. If lipids have unsaturated bonds, they form kinks in the chain structure which disturb the packing structure of lipids and reduce the packing density. Moreover, larger head groups increase the distance between lipids resulting in a bigger area per lipid and therefore decrease the packing density.[59] The properties of lipid bilayers such as fluidity are strongly affected by the packing density of these lipids. The more tight the lipids are packed the less is the fluidity of the lipid bilayer. So lipids with small head groups, long hydrocarbon tails, and no unsaturated bonds have the highest packing density making the lipid bilayer less fluid and resulting in an increase in membrane viscosity and rigidity. It is difficult for lipids with unsaturated bonds in the hydrocarbon chain to pack tightly and therefore they form disordered phases in the membrane.

Aside from phospholipids, sphingomeylin and glycosphingolipids are the most well known lipids in mammalian cells membranes that because of their structure can pack tightly and form a solid gel phase. The solid gel phase made by tightly packed sphingolipids is fluidized by sterol lipids, mainly cholesterol, to form a liquid ordered phase. Cholesterol is the main sterol lipid in the cell membrane that is believed to make clusters with sphingomeylin in the cell membrane that are called lipid rafts.[60, 61] It has been suggested that these clusters are crucial for cells in terms of localization of the SNARE complex that is required for fast and efficient vesicle docking with the cell membrane.[60, 62, 63, 64, 65] Several studies have shown that disturbance of these domains mainly by depleting cholesterol can suppress exocytosis and one explanation has been the re-localization of SNARE proteins that are crucial for the exocytosis process.[60, 62, 64]

Another property of the lipid is curvature that is determined by the size of the head group in relation to the size of the tail group. In general, phospholipid structures can

be cylindrical (no curvature) like the phosphatidylcholines (PC) where the head and tail group are about the same size, conical (negative curvature) like PE where the head group is relatively small, or inverted conical (positive curvature) like the lysophosphatidylcholines (LPC) where the tail group is relatively small compared to the head group (Fig. 5). Mixtures of lipids with different molecular geometries induce a curvature stress in the cell membrane that makes it possible for the cell to go through different shape changes during the exo- and endocytosis process. It is worth noting that membrane proteins can also produce membrane curvature through three different mechanisms including 1) scaffold mechanism, 2) local spontaneous curvature mechanism, and 3) bilayer- couple mechanism.[66]

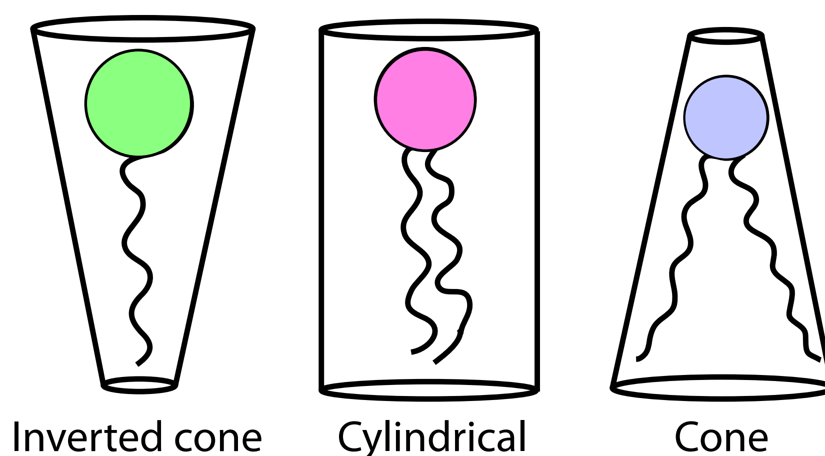

Figure 5. Different lipids with different structures and different curvatures are illustrated.

Membrane Properties are Important in Exocytosis

During the exocytosis process, the synaptic vesicle and cell membrane go through membrane shape changes that involve several high curvature transitions. During the initiation of release, secretory vesicles fuse with the plasma membrane and form an initial fusion pore that either fully dilates and the vesicle releases its entire content, or widens and the vesicle releases part of its content before re-closing. These highly curved structures are essential to the release process and therefore, membrane properties play a very important role in vesicle fusion and pore formation and expansion. Since the lipid composition of the cell membrane can affect the biophysical properties of the membrane including membrane rigidity and curvature, it is a very important factor in regulating and affecting the exocytosis process.[46, 67, 68, 69, 70] Hence, there is an increasing appreciation of the importance of the lipids to various aspects of exocytosis.

In order for the membrane to go through different shape transitions, the membrane needs to bend and stretch, two processes that require high energy and the lipid composition of the membrane can either decrease or increase the amount of energy membrane needs to bend and stretch and this way either facilitates or hinders the exocytosis process. For instance, lipids with different intrinsic curvature can affect the process of exocytosis differently by their positioning in the lipid bilayers. During the initial step of fusion pore formation that the inner leaflet of the cell is positively curved, lipids that promote positive curvature like LPC can facilitate this structure. Meanwhile, the outer leaflet is negatively curved and can be facilitated with lipids that promote negatively curved structures such as PE. This means that the rate of exocytosis can be changed depending on the lipid composition of the membrane and this has been shown in several studies done in single cell experiments using secretory cell models for exocytosis.[53, 69, 71, 72] In the first paper of this thesis it has been shown that the fusion pore can be affected by lipid curvature and high curvature lipids such as PE are sorted into the high curvature region of the lipid nanotube where they facilitate tube membrane curvature by lowering the resistance to membrane bending.

Aside from membrane curvature, membrane rigidity is also very important in the exocytosis processes and that is also dependant on the lipid composition of the membrane. For instance, in a fluid bilayer cholesterol enhances the membrane mechanical stiffness as the bulky steroid part of the cholesterol embeds into the membrane and interacts with lipid tail groups, resulting in higher packing density of lipids and consequently an increase in membrane bending rigidity.[73, 74] This makes it harder for the membrane to deform and therefore adds energetic resistance to go through high curvature shape deformations, affecting the kinetic of release. In the third paper of this thesis I used artificial cell models to show that cholesterol affects the biophysical properties of the membrane and consequently the exocytosis process.

Another property of lipids that can change the rigidity of the membrane is the lipid charge. If a charged lipid is incorporated into a membrane, it increases the rigidity of the membrane. There have been several studies to investigate the effect of charge on bending rigidity of the membrane and they have shown that increasing the fraction of charged lipids in the membrane results in stronger repulsion in the bilayer plane, for instance between the polar groups of charged lipids.[75, 76] In a fluid membrane where individual components can move freely, if a charged lipid is added to the membrane other charged lipids can move toward or away from the interaction zone in order to minimize the electrostatic energy in response to differences in the charge distribution.[77] This strongly affects the membrane undulations, which is a wavelike movement of the membrane and consequently increases the membrane rigidity and adds resistance to membrane deformation.[75] There are several approaches to measure the bending rigidity of the membrane including analysis of thermal fluctuations of the

membrane and techniques to measure the force applied to bend the membrane using micropipettes, optical tweezers and electric or magnetic fields.

Liposomes as Model Systems

Synthetic vesicles known as liposomes are spherical lipid bilayers with a size distribution from 40 nm up to hundreds of micrometers in diameter. The principle underlying liposome formation from soluble lipids in an aqueous environment is based on the hydrophobic effect. The high hydrophobicity of the lipid tail group results in formation of a closed bilayer shell. Here, the tail groups are facing each other leading to a reduction of free energy of the system. For many lipids, formation of liposomes is more favorable compared to formation of a flat lipid bilayer as in lipid bilayer the tail groups of lipids that are located on the edges are facing the water and this increases the energy of the system.

Liposomes are promising tools in biosensor studies where they can act as a support platform for different molecules such as membrane proteins where the 3D structure of the liposome provides an environment that permits protein flexibility and movement while avoiding direct exposure to the surface.[78] Moreover liposomes have been used extensively in different studies to make lipid bilayers and they can have many different properties depending on their lipid composition, size, surface charge, and method of preparation. It should be also mentioned that liposomes have been extremely useful tools in delivering drugs to different parts of the body and many studies have been devoted to this area.[79]

Formation of Supported Membranes

Although investigations of cell membrane properties using live cells are valuable, the cell membrane is very complicated and a bottom up approach where cell membrane is replaced by supported lipid bilayers can provide a very useful platform for biological studies. A highly used approach to make bilayers is via the fusion of vesicles to a substrate that is very reliable and does not need sophisticated equipment. This method was first introduced by McConnell and co-workers[80] and later has been used by many groups to make supported bilayers on different substrates from various lipids compositions.

The process of vesicle adsorption to a target surface is dependent on having adhesion energy that is favorable and bending energy that is unfavorable. In a theoretical model introduced by Seifert and Lipowsky, it has been shown that bilayer formation is dependent on many factors including lipid composition of the vesicle that can give

different bending modulus and curvature along with the properties of the substrate that results in different adhesive interaction between the vesicle and the solid surface.[81, 82, 83] The size of the vesicle has been also shown to play an important role in bilayer formation in a sense that only vesicles larger than a certain size will rupture after absorption on the surface. For PC vesicles, this size has been calculated to be around 75 nm.[84]

In general, the mechanism of bilayer formation is composed of three stages where at first liposomes are adsorbed at the surface and fill the surface and then at certain vesicle coverage some vesicles rupture leading to a surface with three distinct areas including areas of intact vesicles, bilayers, and naked surface. In the next step a process known as edge induced vesicle rupture drives the rest of the vesicles to rupture. Therefore a supported bilayer is formed that covers the whole surface.[85] There are still many unanswered questions regarding this process including the factors that initiate the rupture and the effect of ruptured vesicles to induce the rupturing of other vesicles. Many studies have been devoted to answer these questions.

In a study done in Fredrik Höök´s lab, four possible pathways have been proposed for the initial rupturing of vesicle on the surface. In the first pathway, the initial pore forms where the membrane is in contact with the surface which then expands resulting in complete collapsing of the vesicle where the outer monolayer is exposed to bulk medium (Fig. 6a). In the second pathway the factor of curvature has been brought into consideration and it has been proposed that the rupture starts from the edge where vesicle has the highest curvature (Fig. 6b). In this pathway, in contrast to first pathway, the outer monolayer is predominantly exposed to the surface. The other two pathways are variations of pathway two where the initial opening happens at different regions of the vesicle membrane.[85]

In another study, a single vesicle assay based on two-color fluorescence microscopy has been used to investigate the steps of bilayer formation.[86] Vesicles were labeled with Texas Red (TR) bound lipids and fusion of these labeled lipids to unlabeled vesicles was observed by dequenching of the TR. Vesicles were also filled with soluble carboxy fluorescein dyes (CF) to observe the rupturing of vesicles on the surface. From these studies, they have proposed four different pathways for bilayer formation including: (1) primary fusion where vesicles first fuse with other vesicles and then is followed by consequent rupture, (2) fusion and rupture occur simultaneously, (3) there is no fusion and therefore no dequenching happens and vesicles rupture at the surface and results in a loss of fluoresence signal from both dyes at the same time, and (4) the vesicles undergo pre-rupturing and the content is lost spontaneously on the surface.[86] The interesting finding from this study was that, depending on the amount of TR incorporated into the vesicle membrane, the percentage of vesicles undergoing pre-rupture increases meaning that the excited

fluorophore facilitates the initial pore formation. In the last paper of this thesis I have focused on the effect of excited fluorophore on initial pore formation in the mechanism proposed for vesicle opening during vesicle electrochemical cytometry.

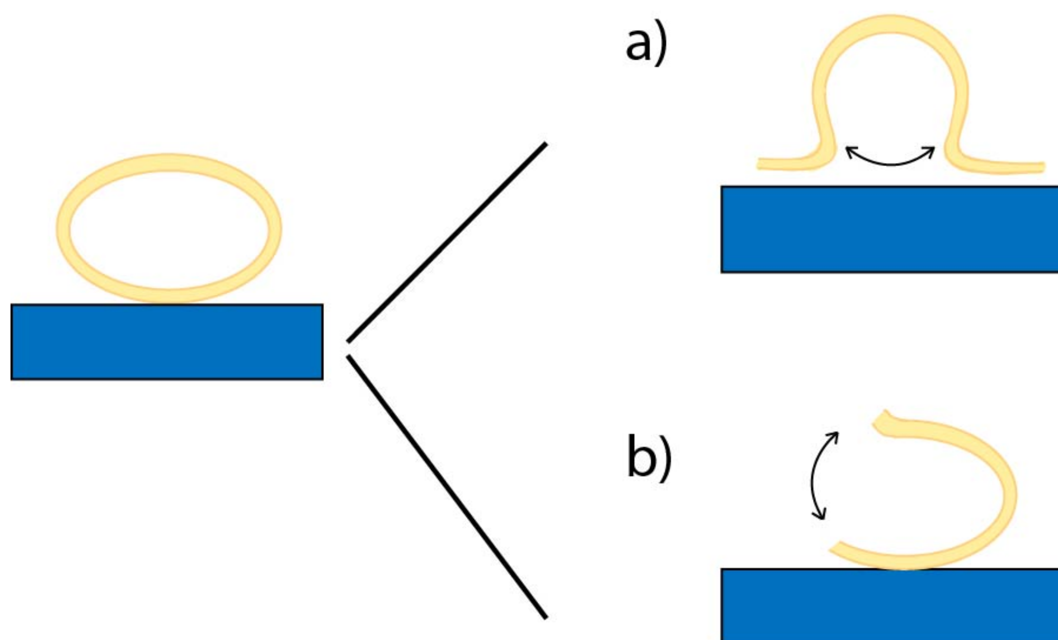

Figure 6. This figure Illustrates the two main mechanisms of liposome rupturing at the surface proposed by Höök and co-workers. a) The initial pore forms where the membrane is in contact with the surface, which then expands resulting in complete collapse of the vesicle, b) the rupture starts from the edge where vesicle has the highest curvature.

Chapter 3

Methods to Study Exocytosis

The broad understanding of neuronal communication that we have today has been achieved through use of many different techniques developed over the last several decades. Each of these techniques, in comparison with each other provides some advantages and limitations. In this chapter some of these techniques are introduced and some of the advantages and disadvantages for each of them are discussed.

Imaging

Imaging techniques can provide very useful information regarding neuronal communication including vesicle trafficking, docking and fusion. Depending on which imaging technique is employed the spatial resolution is different. There are many different imaging techniques including fluorescence microscopy, confocal fluorescence microscopy[87], total internal reflection fluorescence microscopy (TIRFM)[88], stimulated emission depletion microscopy (STED),[89] and also transmission electron microscopy (TEM) that have been used to study the exocytosis process.[90] All these techniques can provide good spatial resolution but the temporal resolution is generally poor compared to the time of an exocytosis event.

Fluorescence Microscopy to Measure Exocytosis

Fluorescence microscopy has been used extensively to monitor exocytosis, endocytosis and vesicle trafficking inside cells.[91] The underlying principle of this technique is to label different intracellular components that are involved in exocytosis processes such as the vesicle membrane itself or membrane proteins of synaptic vesicles (Fig. 7 a, b). In order to label the vesicle membrane, different fluorescent styryl pyridinium molecules composed of lipophilic and divalent cation groups can be used.[91] One of the most widely used fluorescent styryl dyes is FM1-43. FM1-43 is used to label the cell membrane and since these dyes are charged at physiological pH (7.4) they cannot pass through the membrane and therefore only the outer leaflet of the membrane is stained. After the cell undergoes exocytosis the dye that exists in the solution around the cell is incorporated into the membrane of the internalized vesicle easily and reversibly by the endocytosis process allowing tracking of the vesicle. This approach can also provide quantal information since the amount of FM dye incorporated into the membrane will be proportional to the membrane exposed to dye

solution during endocytosis. This method has been used in different studies to provide useful insights about neuronal release including the time course for a complete vesicle cycle, and also to show which mode of release is more prevalent; full or partial release.[87, 92, 93]

The pH-sensitive fluorescent proteins are another class of dyes that can be used to measure vesicular release in real time by monitoring changes in fluorescence signal as a function of vesicular pH during exocytosis. One class of pH sensitive proteins is called the pHluorins. These are genetically engineered from enhanced green fluorescent protein (EGFP). When a synaptic vesicle that has pHluorin tagged to its proteins fuses with the cell membrane, the vesicular solution at pH 5.5 mixes with the extracellular solution at pH 7.4 and the change in pH results in a strong fluorescent signal as the GFP-mutant proteins sense the difference in pH.[94] The principle behind this method is that the fluorescent signal at low pH inside the vesicle is quenched and when the vesicle fuses with the membrane and the pHluorin is released to the pH 7.4 outer solution, a more intense fluorescent signal is observed. There are many studies that have used these pH sensitive proteins to study the kinetic of synaptic vesicles recycle.[94, 95]

Another approach to label the synaptic vesicle is to encapsulate the dye inside the vesicle (Fig. 7c) and for this purpose quantum dots (Qdots) and acidotropic dyes are mostly used. It has been shown that Qdots can be encapsulated into synaptosomes through endocytosis and later be used to study the mode of release.[96] Another way to stain vesicles is to use acidotropic dyes that are neutral at pH 7.4 so they can diffuse through the membrane of synaptic vesicles, but then at low pH inside the vesicle they become charged and therefore concentrating and labeling the vesicle compartment. In a study done by Steyer et al., acridine orange has been used to label the vesicles of chromaffin cells in order to monitor the movement of single vesicles in the cytoplasm through the process of docking, priming, and full exocytosis.[97]

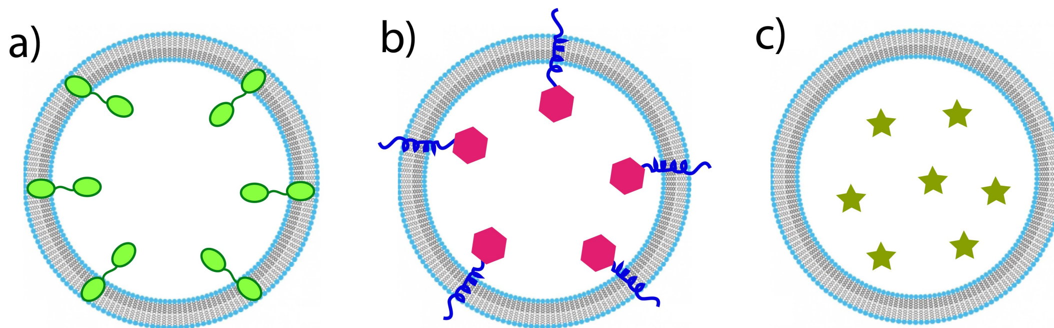

Figure 7. Illustration of different fluorescence methods used to study vesicular release. a) Styryl dye molecules that are placed into the inner membrane of the vesicle during endocytosis. b) pH-sensitive fluorescent proteins such as pHfluorins that are fluorescently quenched inside the vesicle at low pH and show increased fluorescence signal when they are exposed to the higher pH of extracellular media during exocytosis. c) Acidotropic dyes that are neutral at pH 7.4 so they can diffuse through the membrane of the synaptic vesicle but then at the low pH inside the vesicle they become charged and therefore concentrate and label the vesicular compartment.

Total Internal Reflection Fluorescence Microscopy

It was first proposed by E.J. Ambrose that in order to study the contacts formed between cells and solid substrate, it is possible to use the slight penetration of light waves into the less dense medium when totally internally reflected at the glass water interface.[98] This idea was further developed by Daniel Axelrod and called Total Internal Reflection Fluoresence Microscopy (TIRFM), where he used this technique to see the membrane and underlying cytoplasmic structure at the cell-substrate contacts for two types of cell culture (rat primary myotubes and human skin fibroblasts).[88]

In the TIRF microscope, the light is shined at an angel of reflection that is greater than the critical angel and therefore the light beam is totally internally reflected and only one component of the electromagnetic wave, known as evanescent wave, can penetrate perpendicular to the surface with a certain intensity that decays exponentially with distance from the surface (Fig. 8). As the intensity of the evanescent wave decreases from the interface only fluorescent molecules located close to the surface (within around 100-200 nm)[99] can become excited and emit fluorescence, and molecules at further distance are not excited. Therefore using TIRF makes it possible to decrease the background noise and increase the resolution to a couple nm of depth, which is not possible with fluoresence microscopy where light excites fluorophores over a larger depth. This makes TIRF applicable to monitor the cytoskeletal structure of the cell in the contact region without interference from deeper structures and is ideal for the study of the exocytosis process as this occurs at the membrane surface and vesicles are often smaller than 100 nm. Applying TIRF

makes it possible to resolve single exocytotic events when synaptic vesicles are labeled with fluorescent dye such as acidotropic dyes. This approach has been used to study the vesicle movement and location before and during fusion,[97, 100] and also different release mechanisms of exocytosis.[101]

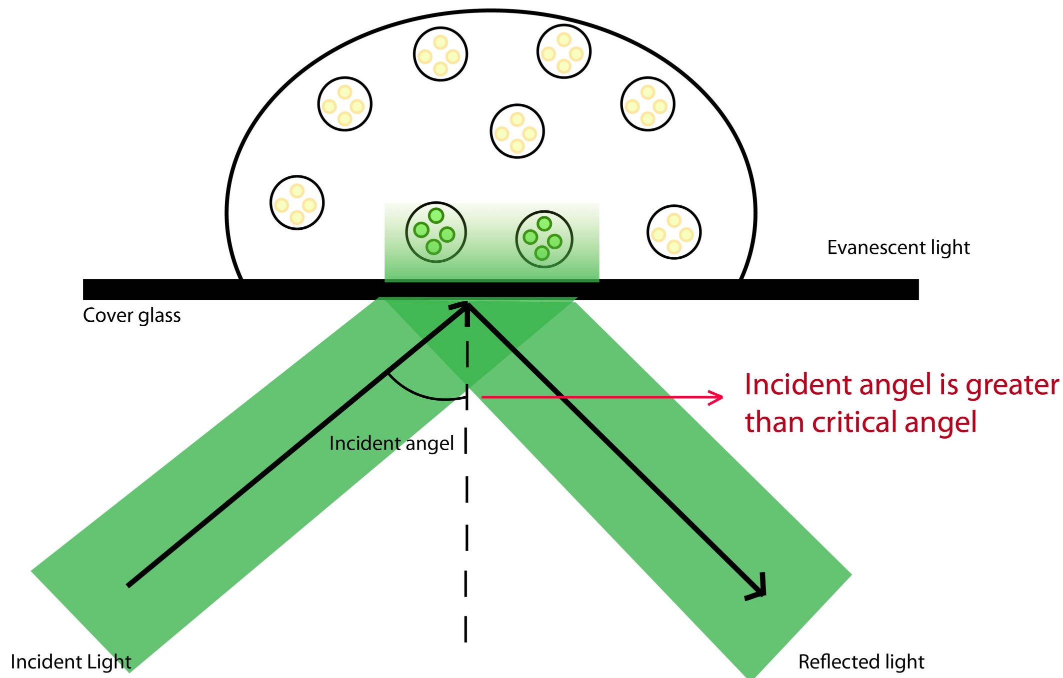

Figure 8. The principle of TIRF Microcopy is shown in this figure. A light beam is directed at the glass-liquid interface at an angle where it is totally internally reflected and only a portion of the light beam, known as the evanescent wave passes the interface to a distance of about 100- 200 nm from the surface, dropping exponentially. The fluorescence is imaged with the microscope.

Transmission Electron Microscopy (TEM)

Applying transmission electron microscopy (TEM), where light is replaced with an electron beam, single vesicles can be resolved and therefore very detailed images from within cells can be obtained. TEM is a widely used technique to study exocytosis since it can give information about the ultra structure of the cell, the size and density population of secretory vesicles in cells, and also can be used to show pharmacological effects on vesicles after treatment with different drugs. TEM has been used to reveal many structural aspects of exocytosis including images of dense core vesicles swelling before fusion pore formation or the Ω-shaped forms of fusion pores that have been applied to calculate the fusion pore size.[90] The only limitation with this technique is that the cells need to be fixed before imaging so real time information cannot be provided and also sample fixation can damage the sample and might result in changes in its structure.

Patch Clamp Measurements of Exocytosis

The patch clamp technique was initially developed by Neher and Sackman in 1976 to investigate ion channel conductance in cells.[102] In this technique an electrode is placed in a glass micropipette filled with an electrolyte and the glass micropipette is positioned into or on the cell membrane to measure the current through the membrane ion-channels while the potential is clamped across the membrane (Fig. 9a). In this way, opening of every individual ion channel can be detected.[103] Patch clamp can be employed to measure not only the membrane conductance but also membrane capacitance.[43] Membrane capacitance is related to the surface area of the membrane and therefore an increase in the total surface area of the cell membrane results in an increase in membrane capacitance. Hence, during the exocytosis process when a vesicle fuses into the cell membrane, the subsequent increase in the surface area of the cell results in an increase in membrane capacitance that can be measured by the patch clamp technique (Fig. 9b). Therefore, single exocytosis events can be recorded. Patch clamp can also be applied to study the endocytosis process since during endocytosis the cell membrane retrieval back results in a decrease in total cell membrane surface area and consequently a decrease in membrane capacitance. This technique has high temporal resolution, which makes it a valuable technique to measure the dynamics of individual vesicle fusion and fission and also to estimate the fusion pore size. However the identity and amount of neurotransmitters released during single exocytosis events cannot be determined with this technique as there is no chemical selectivity.

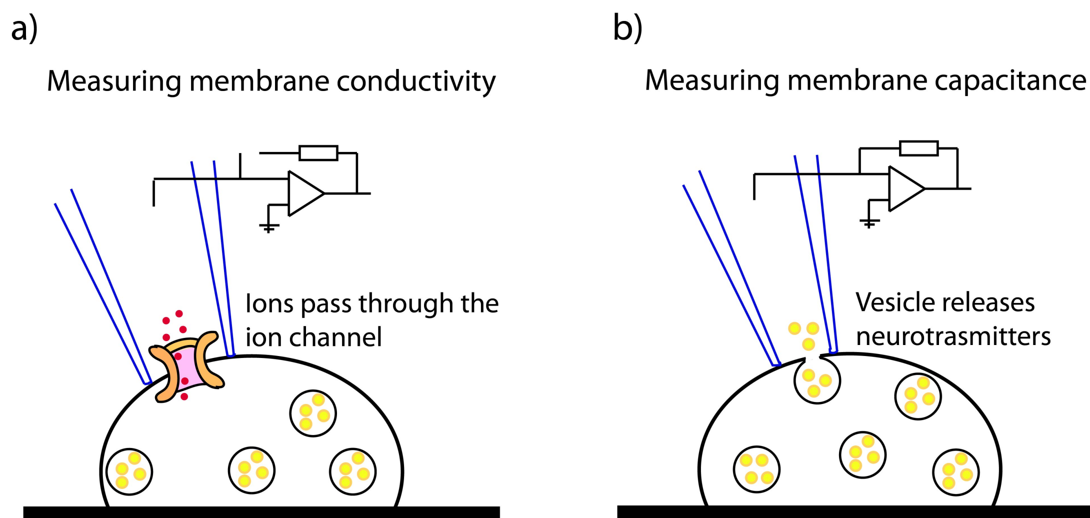

Figure 9. The patch clamp technique can be used to study a) membrane conductance by measuring the current through membrane ion-channels or b) the vesicular release through measuring the membrane capacitance.

Electrochemistry Approaches to Measure Exocytosis

Electroanalytical methods have been used widely to study exocytosis.[104, 105, 106] Several neurotransmitters used in neuronal communication are electroactive (e.g., dopamine, epinephrine, norepinephrine, histamine, serotonin, various neuropeptides, etc.) and they can be oxidized or reduced at the surface of the electrode by applying a potential usually in the range of 0.2-1.0 V versus a Ag/AgCl reference electrode.[106] There are many different electrochemical techniques used to measure exocytosis, with the most common being amperometry and fast scan cyclic voltammetry (FSCV). Both of these techniques are carried out with the use of a reference electrode, a working electrode, and a potentiostat to apply the desired potential to the working electrode. A current amplifier is used to measure the current at the surface of the working electrode during the electrochemical reaction. Carbon fiber electrodes are the most common working electrodes to measure exocytosis which offer many advantages, with the most important being their small size and flexibility.[107]

The use of electrochemistry has one huge advantage over other methods to measure or monitor exocytosis, it is quantitative in that, in the case of amperometry, it can be used to measure the number of molecules released for each event. No other approach does this. The biggest limitation to the use of electrochemical techniques is the fact that they are only applicable to electroactive molecules. There are many studies devoted to overcome this problem by manipulating the electrode surface with a suitable enzyme for a given analyte in order to create a reaction that produces electroactive species at the surface of the electrode.[108] This area has not yet become reality, but has future promise.

Fast Scan Cyclic Voltammetry

In cyclic voltammetry the potential is changed in a triangular pattern. The potential starts from a range where oxidation of the analyte of interest does not occur and is scanned linearly with time to the potential where the analyte starts to oxidize and then to a potential where the oxidative current is diffusion limited and thus drops with time. The oxidation current at the peak is typically measured. Then the potential is reversed and scanned back to the starting potential and the reverse potential results in reduction of analyte that has been oxidized and is still present on the surface of the electrode. The reduction current peaks and then decreases as the oxidized analyte is used up and this peak current is measured. Since each molecule has its own specific oxidation and reduction peak potential, determined thermodynamically, this technique is a great tool to identify the molecule of interest. By cleaver use of the reverse potential, one can even discriminate for instance, between adrenalin and noradrenalin in chromaffin cells.[109] The time resolution of this technique is relatively low

compared to amperometry since data collection is dependent on scan rate and waiting time between scans. Therefore FSCV is not capable of providing kinetic information in terms of opening and closing of the fusion pore and exocytotic release that happens on a millisecond time scale.[109, 110] Thus, FSCV is used most often to identify what is released or to characterize the electrochemical reaction, but amperometry is more often used for quantification of exocytosis release.

Amperometric Measurement of Exocytosis

When it comes to the study of single exocytotic events, amperometry can be used to provide both useful kinetic information and also to quantify the amount of molecules released in single vesicle release events.[104, 111, 112] Amperometric detection compared to FSCV has significantly higher temporal resolution and one can perform recordings on a submillisecond time scale. This high temporal resolution together with the quantitative information makes this method the popular method for studies that involve recordings of single exocytosis events at secretory cells when the released messenger is electroactive. The first study using amperometry was published by Wightman and co-workers where they used this technique to measure exocytosis at catecholamine-containing bovine adrenal chromaffin cells.[104] Following this seminal work, the amperometry technique has been used extensively to study release from different secretory cell models including cells from the brain,[111, 113] peripheral cells,[114] and also nerve cells[115] to answer questions regarding the vesicular release in real time and reveal information regarding different modes of exocytosis such as flickering fusion pores[48] or kiss- and- run exocytosis.[45]

Amperometric measurements of exocytosis at secretory cells are performed by placing an electrode in close proximity to the cell plasma membrane and at the site of release (Fig. 10). A constant potential is held at the electrode surface and the oxidation of the neurotransmitters of interest occurs as they are released via exocytosis against the electrode. Neurotransmitter molecules that are released through exocytosis result in an amperometric spike (Fig. 10). When the vesicular content is released, the amperometric current spike provides both kinetic and quantitative information of molecules released with millisecond time resolution. This time resolution is fast enough to characterize the vesicle fusion process in terms of initial fusion pore formation, fusion pore dynamics and the mode of exocytosis release. Kinetic information that is often used to characterize single exocytotic release events includes rise time (t_{rise}), representing the opening of the fusion pore; the spike half width ($t_{½}$), that defines the duration of the vesicle release event; and spike fall time (t_{fall}), that relates to the closing of the fusion pore. Moreover, by measuring the area under each amperometric spike to obtain the charge (Q) one can calculate the number of moles of molecules (N) released during every single event using Faraday's Law (Q

= nNF), where n is the number of electrons exchanged in the oxidation reaction and F is Faraday's constant (96,485 Coulombs/mole of electrons).

Another feature of the amperometric spike is the pre-spike foot, observed for a fraction of events that appears as a small increase in current directly before the current peak. This current is related to the leakage of neurotransmitters through the fusion pore formed in the initial stage of vesicle fusion.[45, 67, 68, 116, 117] The shape of a pre-spike foot varies greatly, but it can be roughly categorized into two main shape features. The first category of a foot displays a slowly increasing current prior to the sharp rise of the current spike. This foot is referred to as a ''ramp'' foot. A second category has an increase in current followed by a stable current plateau leading up to the current spike. This type of foot is referred to as ''ramp+plateau'' foot. In the extended kiss-and-run or open and closed exocytosis mode, post-spike feet have also been observed where during the process of closing the fusion pore, neurotransmitter leakage from the pore structure is recorded before full closure of the pore and displayed as a current. The recorded shapes of post-spike feet demonstrate the same shapes as for pre-spike feet and can therefore also be categorized into ramp and ramp+plateau shapes.[46, 67, 68] The duration and amplitudes of these feet reveal the activity and the stability of the lipidic pore structure. In the third paper of this thesis, using a high tension plasma membrane cell model, we have clearly shown that high membrane tension along with membrane rigidity can influence the fusion pore dynamics and stabilization resulting in the observation of post spike feet.

In Contrary to FSCV, amperometry is not able to distinguish between different analytes. Another limitation of the amperometry technique is its poor spatial resolution. This problem can be resolved by use of arrays of smaller electrodes to cover the cell and these multiple electrodes can be used to monitor the spatial distribution of neurochemical release across a single cell.[118, 119, 120] Using microelectrode arrays (MEAs) has revealed the presence of "hot spots" or active zones on cells where vesicular release is happening mostly from these regions.[121]

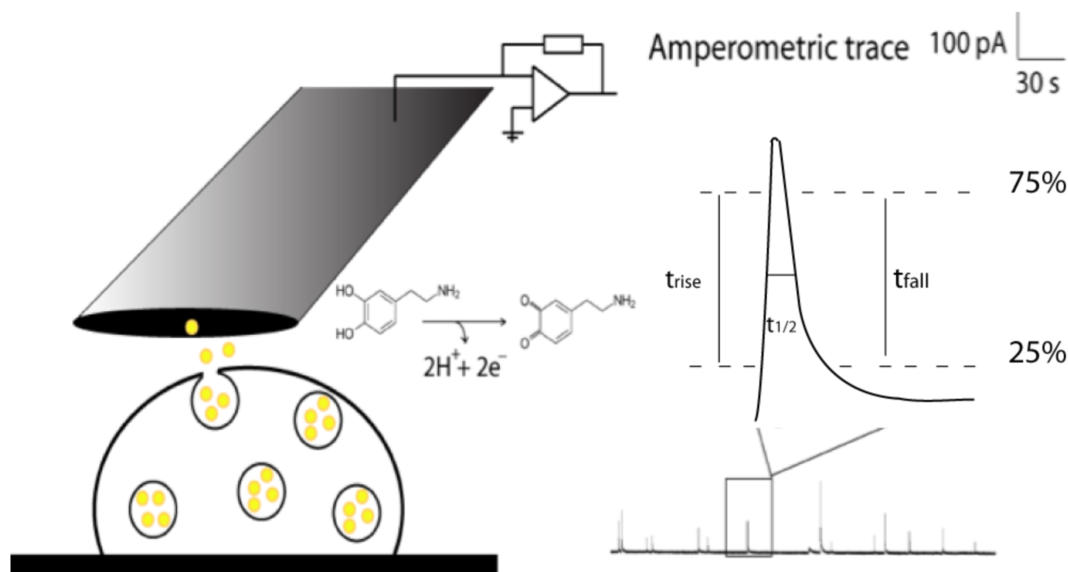

Figure 10. This picture illustrates the placement of an electrode on top of a cell during single cell amperometry recordings (left) and the different temporal aspects of exocytosis measured by amperometry (right).

Electrochemical Cytometry to Measure Vesicle Contents

As mentioned earlier, the dominant mode of exocytosis is partial release where each vesicle releases part of its content and the released amount can be changed by manipulating different factors such as temperature,[50] osmolarity,[54, 122] Ca^{2+} levels,[123] etc. The important question that needs to be addressed here is what percentage of vesicle content is released during the exocytosis process. Therefore a great deal of work has been devoted to developing a technique that can quantify the vesicular content.

Electrochemical cytometry is a method developed in the Ewing laboratory that made it possible to quantitatively probe the vesicular content of an individual synaptic vesicle. Vesicles are typically isolated from a cell or cells through homogenization to break the cell membrane mechanically and then analyzed. The first electrochemical cytometry system was composed of a capillary-microfluidic device, lysis buffer and polarized carbon fiber electrode at a potential sufficient to oxidize the analyte of interest. In the first step, the capillary-microfluidic device was utilized to separate the vesicles by capillary electrophoresis (CE) and then transport the vesicles to a detection zone where individual intact vesicles were lysed chemically at the electrode surface and the content oxidized resulting in an amperometric spike (Fig. 11). The amperometric spike was then used to calculate the amount of transmitter per vesicle similarly to the approaches described in the amperometry section. This technique was applied to determine the vesicular content of rat pheochromocytoma cells (PC12), and

mouse primary neurons.[44, 124]

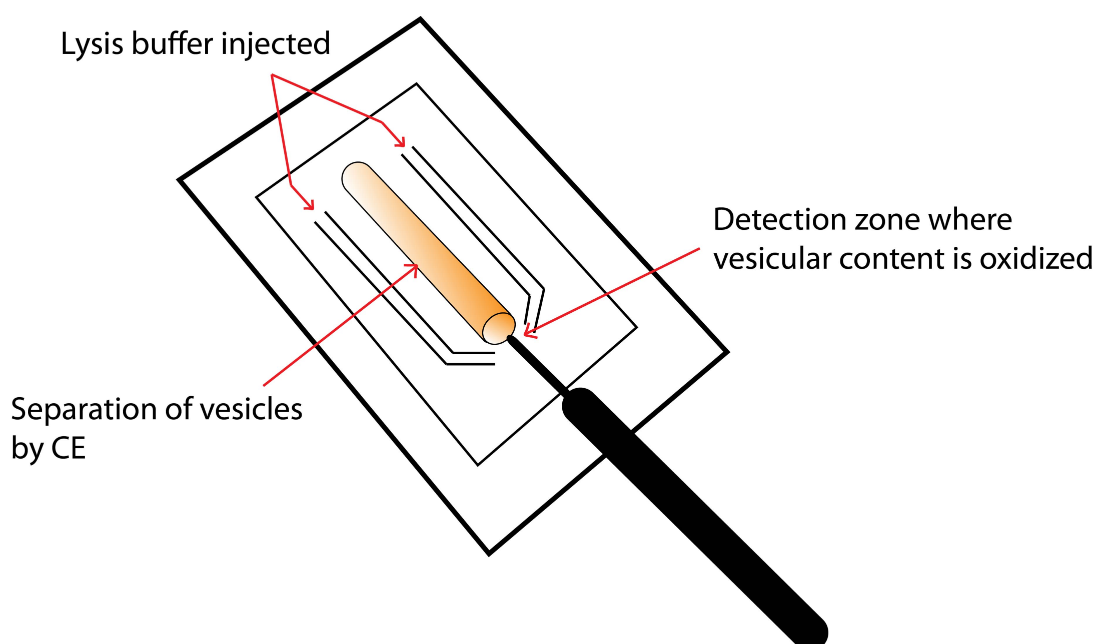

Figure 11. Illustration of the electrochemical cytometry set up where vesicles are separated in a capillary-microfluidic device and transferred to a detection zone at a cylindrical carbon fiber electrode following which they are lysed and the electroactive messenger content oxidized at the electrode surface.

I have recently been involved in developing a new variant of electrochemical cytometry in the Ewing laboratory. This is called vesicle electrochemical cytometry and involves adsorption and subsequent lysis of vesicles onto and then at an electrode surface.[125] This approach does not require the difficult electrophoretic separation step and lysis buffer (Fig. 12).[125] In this technique a 33 µm electrode is placed inside a solution of isolated chromaffin vesicles and vesicles adsorb at the electrode surface and with time some of them undergo rupturing and result in an amperometric spike. The interesting observation is that not all vesicles rupture at the electrode surface and many of them stay intact. We have shown that electroporation is the main cause for initial pore formation. Thus, at higher electrode potentials the number of vesicles that rupture at the surface increases. However, for electroporation to be more potent, the membrane proteins between the electrode and vesicle need to diffuse away allowing the lipid bilayer to come in close contact with the electrode surface. A different approach using synthetic liposomes has been used to remove any effects from membrane proteins as a barrier for electroporation. In comparison with chromaffin vesicles, pure synthetic liposomes rupture more easily on the surface and rupture events occur with duration much faster than that of chromaffin vesicles. The last three papers of this thesis describe the technique and the underlying mechanism of vesicle

adsorption and rupturing at the electrode surface. Moreover this technique has been further developed to where a nanotip conical carbon-fiber microelectrode can be inserted into a cell. Vesicles in the living cell cytoplasm impact and adsorb at the electrode surface followed by rupturing. This technique has been called intracellular vesicle electrochemical cytometry and has been used to quantify the amount of neurotransmitters encapsulated inside PC12 cells vesicles. Findings from this study have also confirmed that only a fraction of vesicular content is released during the exocytosis process.[52]

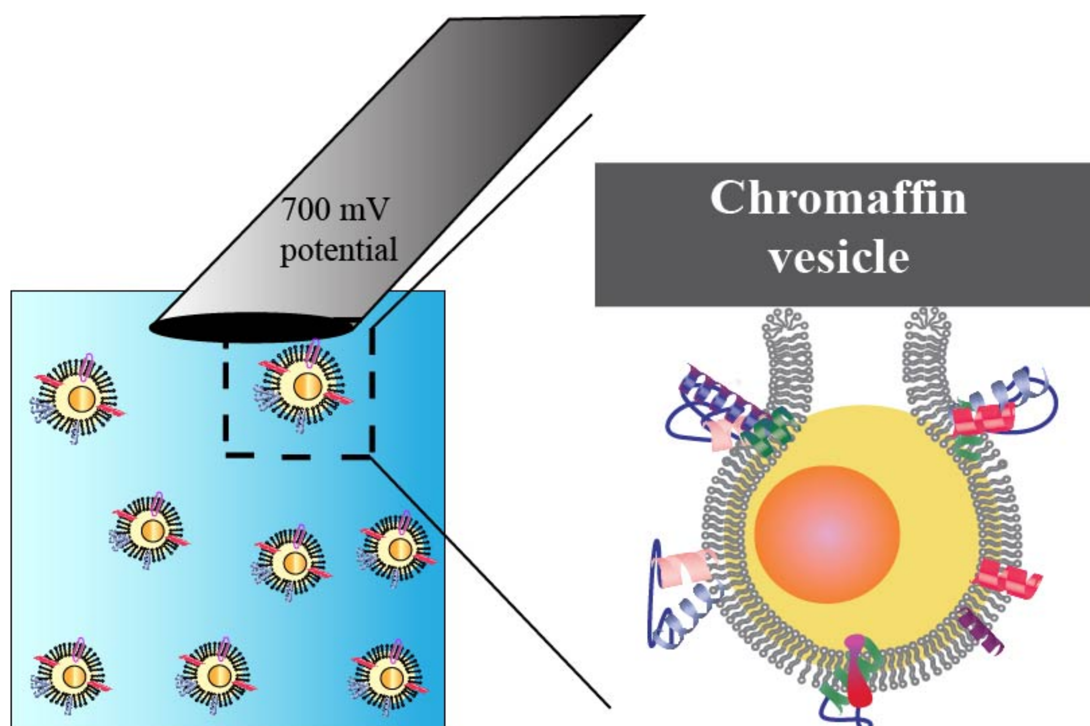

Figure 12. Illustration of vesicle electrochemical cytometry where an electrode is placed in a solution of isolated vesicles. Vesicles absorb at the surface and some of them rupture with time resulting in an amperometric spike.

All techniques mentioned above have some advantages and limitations and there are many studies that have attempted to overcome these limitations by combining some of the techniques together. For instance, in a study done by Becherer et al. the combination of TIRF Microscopy and membrane capacitance recording has been applied to quantify exocytosis from adrenal chromaffin cells.[126]

Chapter 4

Study in Vivo in Animal and Animal Models

Since many animals are similar to humans in terms of genetic and organ function, animal models are being used vastly in neuroscience to answer several questions regarding neuronal communication and neurodegenerative diseases. Some of the mostly used model organisms that have similarities with humans in the nervous system in terms of neurotransmitters, receptors and signaling molecules include the *Caenorhabditis elegans* (nematode worm), *Danio rerio* (zebra fish) and *Drosophila melanogaster* (fruit fly).[127] Many discoveries in this area including the role of neurotransmitters in cell communication have been achieved through animal studies. Even though these achievements are valuable, it is crucial to use alternative models that can be employed instead of animals to develop simple reductionist approaches. Until now different cell cultures and artificial cell models have been developed that can successfully be used to replace animals in some areas of research.

Cultured Cells as Models (Chromaffin Cells and PC12 Cells)

Many different secretory cells such as neuroendocrine cells are useful models to replace animal models in neuroscience research.[128] One of the most common neuroendocrine cells used for studying single exocytosis release event is the chromaffin cell.[129] Chromaffin cells are isolated from the adrenal medulla, a small part of the gland located above the kidneys in mammals. This type of cell has large dense core vesicles and the catecholamine concentration of chromaffin granules of human pheochromocytoma have been reported to be approximately 72% noradrenaline, 27% adrenaline and rest is trace amounts of dopamine.[130] Since these catecholamines are electroactive, many studies have been carried out on this cell type as a model for exocytosis using different electrochemistry techniques.[131] Another live cell used often as a cell model in neuronal communication is the pheochromocytoma (PC12) cell that was first isolated from a rat adrenal pheochromocytoma and published in 1976.[132, 133, 134] This cell has both large dense core vesicles filled with mostly dopamine and small synaptic vesicles that are filled with acetylcholine. Single cell amperometry using microelectrode arrays (MEAs) on both chromaffin and PC12 cells have revealed the presence of hot spots where most of the exocytotic release occurs. These hot spots are believed to represent active zones where the density of Ca^{2+} channels is high.[119, 121]

PC12 and chromaffin cells use the same mechanism as neurons to undergo exocytosis meaning that the same as neurons in these cells SNARE proteins are responsible to dock the vesicle in the membrane and upon stimulation, Ca^{2+} ions enter the cell and release happens through priming and fusion.[1]

Artificial Cell Models

Although performing studies using living cells provides useful information regarding the exocytosis process, their complexity makes it difficult to investigate the effect of different components on the exocytosis process one at the time and therefore a bottom-up approach where one can have better control over the system is very valuable. In the following sections, three different artificial cell models are described.

DNA-Zipper Based Artificial Cell Model for Exocytosis

In this model, the whole process of full exocytosis is mimicked by replacing the SNARE proteins with complementary DNA strands that zip together under the right conditions, hence called a DNA-zipper. These molecules typically are linked to a cholesterol base that allows them to self-insert into a lipid bilayer. SNARE complexes, as explained before, are composed of t-SNARE proteins on the inner leaflet of the cell membrane and v-SNARE proteins on the outer leaflet of the vesicle membrane that hybridize and make a helical structure that brings the membrane of the vesicle close to the membrane of the cell as the protein strands wind together. The same mechanism that allows the SNARE complex to pull the membranes together can be mimicked using the DNA-zippers that use two sets of complementary double stranded DNA. It has been shown that these DNA structures can hybridize in a zipper-like fashion and when complementary strands are placed into the membrane of two different vesicle compartments, vesicle-vesicle fusion in suspension can be induced.[135, 136]

This cell model system is constructed with a giant unilamellar liposome as the artificial cell with one complement of the double stranded DNA attached to the inner leaflet of its membrane imitating the t-SNARE proteins. Small synthetic liposomes are used to mimic the synaptic vesicles, and here the complimentary double stranded DNA is attached to the outer leaflet of their membranes imitating the v-SNAREs. Typically, the giant unilamellar vesicles used are made of soybean lipid extract. First, DNA strands of one complement are injected into the giant unilamellar vesicle using electroporation. Since the DNA strands have cholesterol tails, they partition into the membrane. After this step, small synthetic liposomes filled with electroactive molecules and labeled with the complementary DNA strands in the outer leaflet of the

membrane bilayer are electroinjected inside the giant liposome. By microinjection of Ca^{2+} ions into the giant liposome, fusion of small vesicles is initiated and full exocytosis is mimicked. The small liposomes are filled with electroactive molecules and therefore by placing an electrode close to the artificial cell membrane single vesicle exocytotic release events can be recorded as an amperometric spike (Fig. 13).[137]

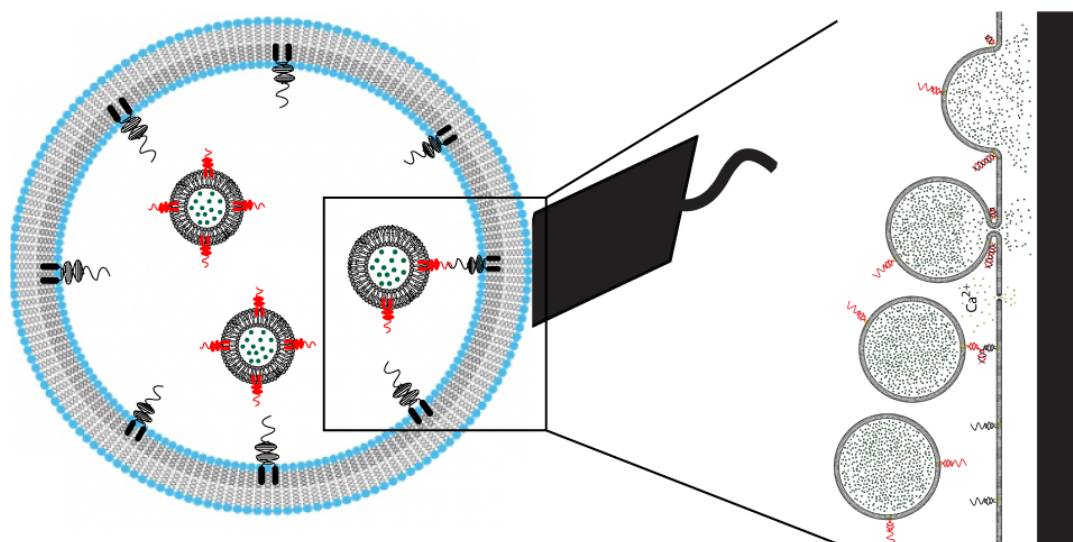

Figure 13. A schematic showing the concept of amperometric recordings of single vesicle release events from the DNA-zipper based cell model for exocytosis. Small synthetic vesicles filled with electroactive molecules and labeled with short DNA strands on the outer leaflet are microinjected into the giant unilamellar liposome that has complementary DNA strands on the inner leaflet of the membrane. The hybridization of DNA strands with the help of ca^{2+} ions docks the vesicles to the giant unilamellar membrane and triggers the fusion of the vesicle with the giant liposome membrane. The release of vesicle content is recorded by the electrode, resulting in an amperometric spike.[137]

Lipid Based Cell Model for Exocytosis

This lipid based artificial cell model is constructed from a giant unilamellar liposome attached to a multilamellar liposome that mimics the later stages of exocytosis. In this model system, the tip of a microinjection pipette filled with a solution of electroactive molecules is pushed through the membrane of a giant unilamellar liposome (GUV) with the help of the electroporation and then through the other side of the liposome. Once the pipette tip exits from the membrane the lipids seal around the tip. When the pipette is pulled back into the center of the artificial cell a nanotube is formed between the tip and the membrane. By applying a constant pressure solution into the lipid nanotube a small daughter vesicle can be inflated inside the artificial cell. This assembly, consisting of a daughter liposome, a nanotube, and a GUV simulates the

secretory vesicle, the fusion pore, and the plasma membrane structure formed during exocytosis. Continuously microinjecting solution enlarges the daughter vesicle until it reaches a critical size where the nanotube is very short. At this stage, the vesicle distends into the artificial cell membrane and releases its content into the extracellular solution, mimicking the final stages of exocytosis. By placing an electrode close the cell membrane, single vesicle release can be recorded as individual amperometric current spikes (Fig. 14). This model system is a low membrane tension system since the artificial cell is attached to a multilamellar liposome that provides a source of lipid for expansion and a sink for contraction. This model has been used before to show that membrane mechanics and dynamics can alone drive the later stages of exocytosis.[138] We have applied this model to investigate the effect of cholesterol on membrane biophysical properties and consequently on the later stages of exocytosis.

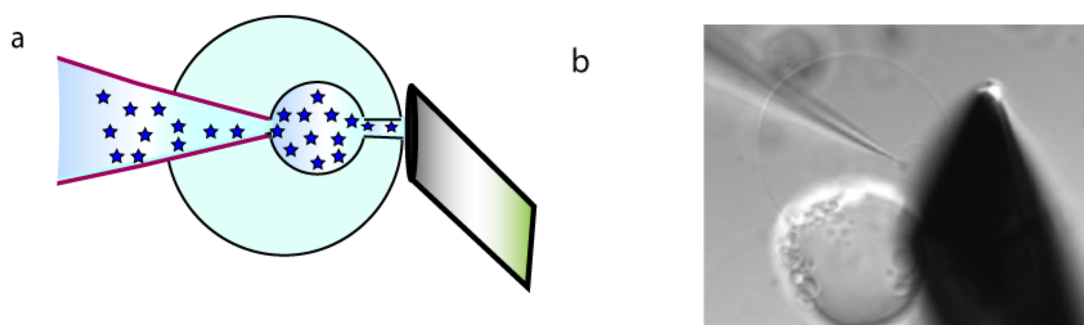

Figure 14. a) A schematic illustrating the principles of the lipid based artificial cell model for exocytosis. When the pipette is pulled back into the liposome a nanotube is formed. By applying pressure to the pipette, a daughter vesicle is formed inside the giant unilamellar vesicle. Microinjection of solution increases the daughter vesicle size and when the vesicle reaches the artificial cell membrane the vesicle fusion pore dilates and releases the vesicle content mimicking the lateral stages of exocytosis.[138] b) A DIC-image of the giant unilamellar liposome cell model, formed from a multilamellar liposome.

Plasma Membrane Cell Model for Exocytosis

The cell model explained above has been further developed to be one step closer to the conditions of a real cell. Here, plasma membrane constructed from PC12 cell plasma membrane vesicle buds, also referred to as blebs, was used to inflate a vesicle on the inside. This model system has been shown to mimic two different modes of release: full and partial vesicle release (Fig. 15). It was shown that one factor that regulates the modes of exocytotic release is membrane tension. The membrane tension in the plasma membrane cell model is significantly higher than in the giant unilamellar artificial cell model, since the bleb membrane does not have any large excess lipid sources connected to the plasma membrane. Full release of the vesicle contents is observed in the giant unilamellar artificial cell model, whereas the partial

vesicle release is only observed in the bleb model system with the higher tension. When a daughter vesicle comprised more that 4% of the entire bleb membrane, at a critical point due to the increase in membrane tension, the nanotube opens into a wider size and the vesicle releases some of its content before collapsing back into a nanotube. This results in a partial release of the vesicle content. This artificial cell model brings us one step closer to the situation of real cell and can be used to study both full and partial release of the vesicle content, possibly corresponding to the full and kiss-and-run exocytosis that is believed to occur in most secretory cells.[139] By observing the effect of membrane tension on regulating modes of release, we further investigated the effect of rigidity on these two modes of exocytosis by manipulating the cholesterol content of the membrane. Our findings show that the cholesterol content of the membrane, or membrane rigidity, plays a significant role in determining the kinetics of vesicle release and also along with membrane tension affects the dynamics of the fusion pore.

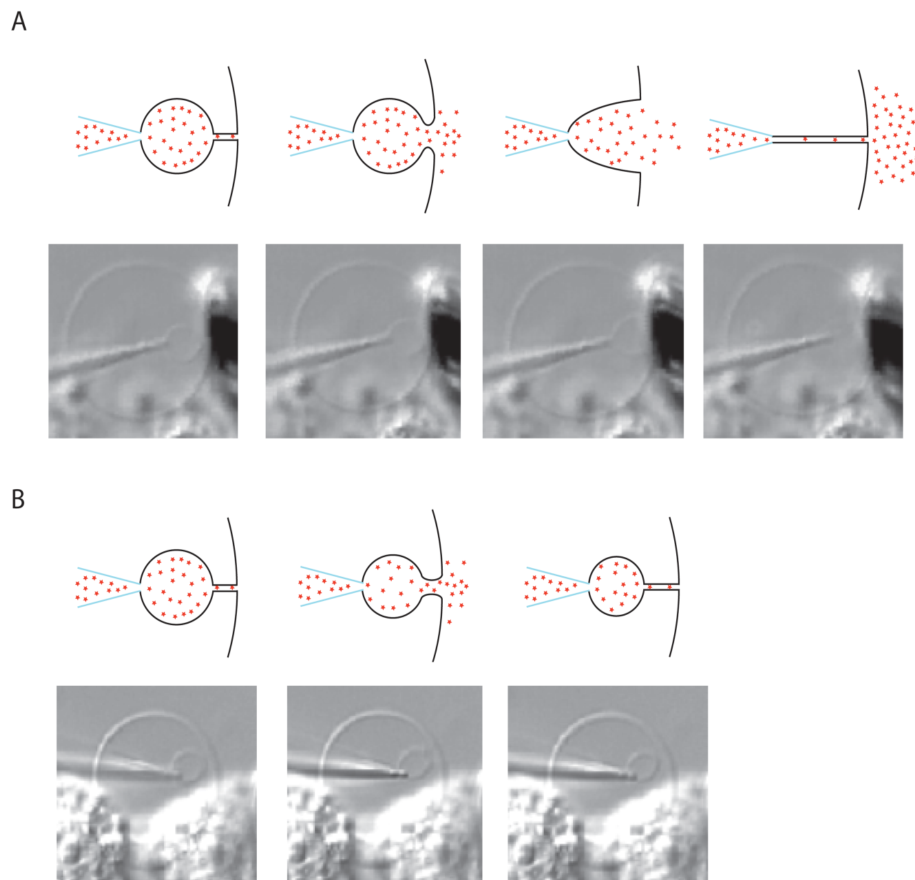

Figure 15. The plasma membrane cell model can mimic two modes of release A) full release where a vesicle is inflated in size until it reaches the giant unilamellar membrane and releases all its content before collapsing back into a nanotube, and B) partial release where owing to high membrane tension the vesicle pore opens up to wider pore releasing some of its vesicular content before collapsing back into lipid nanotube.[139]

Summary of Papers

The general aim of my thesis is to better understand the exocytosis process. My thesis can be divided into two main parts. In the first part, including the first three papers, I have worked with artificial cell models to answer some fundamental questions regarding the exocytosis process. The second part of the thesis includes the last three papers where I have been involved in developing a technique to quantify the vesicular content and I have tried to better understand the underlying mechanism.

Paper I

In this paper, liposome–lipid nanotube networks have been used to study the role of lipids on the formation and dynamics of the fusion pore. These networks are well characterized in terms of the diameter of the giant unilamellar vesicles they are constructed from and the length of the nanotubes connecting them. In this study the effect of different membrane lipid compositions on the dimension of lipid nanotubes has been investigated and altering the lipid composition of the network membrane was carried out either by lipid incubation or lipids were added to the total lipid composition during liposome formation. It has been shown that high curvature lipids such as PE are sorted into the high curvature region of the lipid nanotube where they facilitate tube membrane curvature independent of the method of addition.

Paper II

There have been many studies devoted to understand the factors that determine the mode of release in the exocytosis process. Although findings from live cells are very valuable, the complexity of live cells often makes it difficult to interpret the results. In this paper, working with others, I have successfully developed an artificial cell model constructed from blebbing cell plasma membrane that can mimic both full and partial release and we showed that the switching mechanism for determining the mode of release is membrane tension. These results suggest that the partial distension mode might correspond to an open and closed mechanism as the general form of release from secretory cells.

Paper III

In this paper, I have tried to use the model developed in paper II along with another cell model constructed from pure lipid to investigate the effect of membrane rigidity in the exocytosis process by changing membrane cholesterol content. The results show that with higher membrane cholesterol the kinetics for vesicular release are

decelerated in a concentration dependent manner for both full and partial release. Moreover we have shown that for partial release, enhancing membrane rigidity adds resistance to a dilated pore to re-close. This implies that simply the cholesterol dependent biophysical properties of the membrane directly affect the exocytosis kinetics and the fusion pore dynamics that are related to regulation of neurochemical release.

Paper IV

In this study, a technique called vesicle electrochemical cytometry was developed to quantify the content of individual chromaffin vesicles. In this technique, a 33 μm diameter disk-shaped carbon electrode is placed in a solution of isolated vesicles where they adsorb onto the electrode surface and sequentially rupture at the electrode surface, trapping their contents against the electrode. These contents are then oxidized, and this results in a current (or amperometric) peak that can be used to calculate the number of molecules encapsulated in an individual vesicle.

Paper V

In this paper, the underlying mechanism of vesicle rupture on the surface of a polarized carbon fiber microelectrode during vesicle electrochemical cytometry was investigated. It was shown that electroporation causes the formation of the pore after vesicle adsorption at the polarized electrode surface and it appears that in order for the electroporation to be effective the proteins on the membrane need to move out of the way to allow close contact between the membrane and the electrode surface. In another approach where we used lipid-only transmitter-loaded liposomes instead of native vesicles the rupture events occurred much faster in comparison with native vesicles. It was then showed by incorporating peptide into the liposome membrane that rupture events occur with duration slower than that of liposomes and faster in comparison to native vesicles.

Paper VI

Following paper V, to better understand the mechanism of vesicle rupturing at the surface in vesicle electrochemical cytometry the membrane properties of vesicles was manipulated to change energetics. Here, the membrane properties were affected using excited fluorophores conjugated to lipids, which appears to make the membrane more susceptible to electroporation. The data suggest that by having excited fluorophores in close contact with the membrane, membrane lipids (and perhaps proteins) are oxidized upon production of reactive oxygen species, which then leads to changes in bulk membrane properties and the formation of water defects that make the membrane more fragile to electroporation and increase the fusibility of vesicles at the

electrode surface. Additionally, application of DMSO to the vesicles increases the membrane area per lipid and decreases the membrane thickness resulting in the same enhancement in vesicle opening and supporting the proposed mechanism of vesicle opening with excited fluorophore in the membrane.

Conclusion and Future Outlook

Exocytosis is the key regulatory event in neuronal communication that occurs through the fusion of intracellular transmitter packed vesicles with the plasma membrane. The main aim of this thesis has been to shed light on the exocytosis process and make the underlying mechanism more clear.

The exocytosis process occurs through different modes, which in general can be categorized as full fusion where the vesicle completely collapses with the membrane and releases all its content and partial release where after the formation of the fusion pore the vesicle releases part of its content before the pore re-closes again and the vesicle recycles. Here we have successfully developed an artificial cell model that can mimic both full and partial release and we have shown that the underlying factor that determines the mode of release is membrane tension. We have also shown that by changing the rigidity of the membrane through manipulating membrane cholesterol content we can not only change the kinetics of exocytosis but also the dynamics of the fusion pore in partial release. The most interesting finding here is that by increasing membrane rigidity we have observed post spike feet, as observed in PC12 cells. Having this sophisticated artificial cell model provides a great platform for studies regarding the factors affecting both the mode of release and the kinetic of exocytosis process. The advantage of this artificial cell model is that compared to live cells, membrane proteins involved in the exocytosis process such as SNARE proteins are not functioning and therefore this makes the interpretation of the result less challenging. Studies with live cells have shown that treating cells with different drugs can change the mode of release toward full fusion and this model can be a great tool to investigate whether the underlying mechanism of partial release is a change in the biophysical properties of the membrane or if it is related to membrane proteins. It would also be interesting to incubate the plasma membrane with different lipids with different charges and curvatures that result in changes in membrane properties and to probe if this affects the mode of release and/or the dynamics of fusion pore opening and closing.

An important question that has been addressed in different studies is what percentage of vesicular content is released during partial mode of exocytosis? Electrochemical cytometry was the first technique developed that made it possible to quantify the vesicular content in cells. We have further developed this to create a technique called vesicle electrochemical cytometry using an electrode in a solution of isolated vesicles to measure content from amperometric spikes. This technique provides a great opportunity to study the effects of different chemicals and drugs on vesicular packing efficiency. For example it has been shown than ATP has an important role in keeping

the vesicular content constant and this technique can be utilized to better understand the underlying role of ATP in packing neurotransmitters inside vesicles. Furthermore vesicles prior to measurements can be treated with different lipids or chemicals that change the biophysical properties of the vesicle membrane and the rupture mechanism of vesicles at the electrode surface can be studied, which can give more information about the interaction of vesicle with the surface and the initial pore formation.

In summary the artificial cell models and the techniques that have been presented in this thesis can provide a great platform to better understand the exocytosis process and also the factors that can have different effects on distinct aspects of exocytosis process.

Acknowledgements

When you are close to finishing your PhD you realize that a PhD is not the accomplishment of only one person but a group of people whose support and love is priceless. I would like to acknowledge some of the people who have helped me during this journey and let them know that none of this would have been possible without every single one of them.

I thank my main supervisor **Andrew Ewing** who has always believed in me and with his support, constructive feedback, and great discussions I have grown to be a better scientist.

I thank my co-supervisor **Ann Sofie Cans** who truly is one of the nicest people I have ever met. Her support and help during this 5-year journey has been very valuable.

I want to acknowledge the nicest couple ever, **Lisa** and **Mike**, who are not only great colleagues but also awesome friends. As I have said many times, starting my PhD working with you two was one the best things that has happened to me.

I need to thank my great great collaborators **Soodi**, **Jelena**, and **Johan** for always being there when I needed someone to discuss science or complain about working for the PhD. Good memories that we have made during the last 5 years like heated discussions, working late at nights, and travelling for conferences will always bring a smile to my face when I think about them.

I thank **John** and **Tina** not only for fun times at fikas and afterworks but also for helping me with SIMS experiments.

I thank my awesome friend **Minoo** for proof reading this thesis. I believe that I must have done something great in the past to have such an amazing friend in my life.

And, I thank all my other great past and present colleagues for great discussions in meetings and fun discussions in coffee room. And, I thank all my outside work friends who patiently listen to me whenever I need someone to talk to.

I am eternally grateful to my parents **Ayoub** and **Zohreh**, my beloved sister **Negar** and brother **Amir** who give me the greatest support I could ever ask for. The love that I have received from you has sent me to the moon and back and I will never find the words to express how grateful I am to have you as my family.

And, last but not least, I am supremely thankful to my husband **Mehdi**. We have grown up together and have been through best and worst. A lot of things have changed, but the one thing that has stayed the same is the way you make feel about myself every single day. Having you by my side not only PhD, but all other challenges in life are accepted ☺

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