THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

Investigation of Exocytosis for a New Paradigm of Plasticity in Biological Systems Using Electrochemistry and Mass Spectrometry Imaging

Chaoyi Gu

UNIVERSITY OF GOTHENBURG

Department of Chemistry and Molecular Biology

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Cover illustration: Exocytosis is quantified by single cell amperometry (left), vesicular transmitter content is quantified by intracellular vesicle impact electrochemical cytometry (right bottom), and membrane lipids are analyzed by mass spectrometry imaging (right top).

chaoyi.gu@gu.se

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Department of Chemistry and Molecular Biology
University of Gothenburg
SE-405 30 Göteborg, Sweden

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Abstract

Cellular communication is a vital process and serves as the basis for complex brain functions in multicellular organisms. The majority of cellular communication is achieved via the release of specific signaling molecules, transmitters and modulators, via a process termed exocytosis. Since dysfunction of cellular communication can result in severe outcomes, exocytosis is tightly controlled and regulated. Secretory vesicles are the cellular organelles that serve as functional units to carry out exocytosis. To initiate exocytosis, secretory vesicles need to be docked and primed to the cell membrane. The subsequent fusion between vesicles and cell membrane allows the release of signaling molecules to the extracellular space where they can travel to other cells and transfer a message.

Exocytosis is a highly complicated process and its regulation involves a large number of proteins as well as membrane lipids. To study specific aspects of exocytosis, simplified biological model systems have been developed and widely used, including mammalian cell lines and the invertebrate *Drosophila melanogaster*. A couple of methodologies in the areas of electrochemistry and imaging are available for quantification or visualization of exocytosis. Single cell amperometry (SCA) and intracellular vesicle impact electrochemical cytometry (IVIEC) are two electrochemical techniques that can be used to quantify the number of signaling molecules being released from a vesicle and the number stored inside a vesicle, respectively. Fluorescence imaging is capable of providing spatial information related to exocytosis, including vesicle movements and exocytotic protein machinery, complementing the electrochemical techniques. The role of membrane lipids in regulating exocytosis can be studied using mass spectrometry imaging (MSI).

The main focus of the papers included in this thesis has been to investigate the alterations of exocytosis and membrane lipid composition in relation to cognition and the formation of plasticity. In paper I, the mechanism by which cocaine and methylphenidate (MPH) alter exocytosis as well as vesicular transmitter storage was studied in cells to understand the opposite effects of these two drugs on cognition. Paper II followed the release and storage of neurotransmitters during repetitive stimuli to understand activity-dependent plasticity. A quantitative comparison of SCA measurements between nanotip and disk electrodes was carried out in paper III to support the use of nanotip electrodes for both IVIEC and SCA. In paper IV, the effect of zinc deficiency on membrane lipid composition was examined in *Drosophila*

brains using MSI and the results resemble the changes observed with cognitive-impairing drugs. As a follow-up study of paper II, paper V applied MSI to investigate the alterations of cellular membrane lipids induced by repetitive stimuli and suggested increased membrane curvature as the driving force to stabilize the exocytotic fusion pore. A technique using two nanotip electrodes to simultaneously perform SCA and IVIEC was developed in paper VI, enabling direct comparison between vesicular release and dynamically altered vesicular storage.

The combination of SCA, IVIEC, and MSI has provided useful insights into the mechanism and regulation of exocytosis. The concept of partial release, during which the fusion pore opens and closes to allow only a fraction of the vesicular transmitter storage to be released, has been supported. This is important as it suggests more pathways to regulate exocytosis. Vesicular release, storage, and fraction of release are all potential factors that can be manipulated to alter cellular communication, offering new possible targets for treating neurological diseases as well as understanding plasticity and memory formation.

Sammanfattning på svenska

Cellkommunikation är en viktig process och fungerar som grund för komplexa hjärnfunktioner i flercelliga organismer. Majoriteten av cellulär kommunikation uppnås via frisättning av specifika signalmolekyler, neurotransmittorer och modulatorer, via en process som kallas exocytos. Eftersom dysfunktion av cellulär kommunikation kan leda till allvarliga resultat, kontrolleras och regleras exocytos nogrant. Sekretoriska vesiklar är de cellulära organellerna som fungerar som funktionella enheter för att utföra exocytos. För att initiera exocytos måste sekretoriska vesiklar dockas och primas till cellmembranet. Den efterföljande fusionen mellan vesiklar och cellmembran tillåter frisättning av signalmolekyler till det extracellulära utrymmet där de kan färdas till andra celler och överföra ett meddelande.

Exocytos är en mycket komplicerad process och dess reglering involverar ett stort antal proteiner såväl som membranlipider. För att studera specifika aspekter av exocytos har förenklade biologiska modellsystem utvecklats och använts i stor utsträckning, inklusive däggdjurscellinjer och den ryggradslösa *Drosophila melanogaster*. Ett par metoder inom elektrokemi och bildåtergivning är tillgängliga för kvantifiering eller visualisering av exocytos. Single cell amperometry (SCA) och intracellular vesicle impact electrochemical cytometry (IVIEC) är två elektrokemiska tekniker som kan användas för att kvantifiera antalet signalmolekyler som frigörs från en vesikel respektive antalet lagrade i en vesikel. Fluorescensavbildning kan ge spatial information relaterad till exocytos, inklusive vesikelrörelser och exocytotiskta proteiner, kompletterande de elektrokemiska teknikerna. Rollen av membranlipider vid reglering av exocytos kan studeras med mass spectrometry imaging (MSI).

Huvudfokus för de artiklar som ingår i denna avhandling har varit att undersöka förändringarna av exocytos och membranlipidkomposition i förhållande till kognition och bildning av plasticitet. I papper I studerades mekanismen med vilken kokain och metylfenidat (MPH) förändrar exocytos samt vesikulär neurotransmittorlagring i celler för att avslöja de mekanismer som ligger bakom de motsatta effekterna av dessa två läkemedel på kognition. Paper II följde frisättningen och lagringen av neurotransmittorer under repetitiva stimuli för att förstå aktivitetsberoende plasticitet. En kvantitativ jämförelse av SCA-mätningar mellan nanotip och diskelektroder utfördes i papper III för att stödja användningen av nanotip-elektroder för både IVIEC och SCA. I papper IV undersöktes effekten av zinkbrist på membranlipidkompositionen i *Drosophila*-hjärnor med MSI och resultaten liknar de förändringar som observerats med kognitivt nedsättande läkemedel.

Som en uppföljningsstudie av papper II applicerade papper V MSI för att undersöka förändringarna av cellulära membranlipider inducerade av repetitiva stimuli och föreföll visa en ökad membrankrökning som drivkraften bakom att stabilisera den exocytotiska fusionsporen. En teknik med två nanotip-elektroder för att samtidigt utföra SCA och IVIEC utvecklades i papper VI, vilket möjliggör direkt jämförelse mellan vesikulär frisättning och dynamiskt förändrad vesikulär lagring.

Kombinationen av SCA, IVIEC och MSI har gett användbar insikt i mekanismer och reglering för exocytos. Konceptet med partiell frisättning, under vilken fusionsporen öppnas och stängs för att endast tillåta en del av transmittorerna i en vesikel att frigöras, har fått stöd. Detta är viktigt eftersom det möjliggör fler vägar att reglera exocytos. Vesikulär frisättning, lagring och delvis frisättning av neurotransmittorer är alla potentiella faktorer som kan manipuleras för att förändra cellulär kommunikation, och erbjuder nya möjliga mål för behandling av neurologiska sjukdomar samt förståelse av plasticitet och minnesbildning.

List of publications and contribution report

I. **Combined Amperometry and Electrochemical Cytometry Reveal Differential Effects of Cocaine and Methylphenidate on Exocytosis and the Fraction of Chemical Release**
Wanying Zhu, Chaoyi Gu, Johan Dunevall, Lin Ren, Xuemin Zhou, Andrew G. Ewing
Angew. Chem. Int. Ed. 2019, 58, 4238-4242

Participated in data interpretation, data discussion, and editing the manuscript.

II. **Plasticity in Exocytosis Revealed Through the Effects of Repetitive Stimuli Affect the Content of Nanometer Vesicles and the Fraction of Transmitter Released**
Chaoyi Gu, Anna Larsson, Andrew G. Ewing
Proc. Natl. Acad. Sci. U.S.A. 2019, 116, 21409-21415

Designed and performed the electrochemical experiments, and analyzed and interpreted the data. Participated in designing and performing the fluorescence imaging experiments with A. L., as well as discussing the results. Outlined and wrote the first draft of the manuscript. Edited the manuscript with the other authors.

III. **Comparison of Disk and Nanotip Electrodes for Measurement of Single-Cell Amperometry during Exocytotic Release**
Chaoyi Gu, Xinwei Zhang, Andrew G. Ewing
Anal. Chem. 2020, 92, 10268-10273

Major contribution in designing and performing the experiments, and analyzing and interpreting the data. Participated in data discussion, and designing and discussing the simulation section with X. Z. Outlined and wrote the first draft of the manuscript. Participated in editing the manuscript with the other authors.

IV. **Zinc Deficiency Leads to Lipid Changes in *Drosophila* Brain Similar to Cognitive-Impairing Drugs: An Imaging Mass Spectrometry Study**
Chaoyi Gu†, Mai H. Philipsen†, Andrew G. Ewing
ChemBioChem 2020, 21, 1–5

Participated in designing the experiments with M. P. and performed sample preparation for the mass spectrometry imaging experiments. Contributed to data interpretation and data discussion with the other authors. Outlined the manuscript, wrote the first draft, and edited the manuscript with the other authors.

V. **Mass Spectrometric Imaging of Plasma Membrane Lipid Alteration Correlated with Amperometrically Measured Activity-Dependent Plasticity in Exocytosis**
Chaoyi Gu†, Mai H. Philipsen†, Andrew G. Ewing
Int. J. Mol. Sci. 2020, 21, 9519

Contributed to designing the experiments and preparing samples for the mass spectrometry imaging experiments with M.P. Interpreted and discussed the data with the other authors. Outlined the manuscript, wrote the first draft, and edited the manuscript with the other authors.

VI. **Simultaneous Detection of Vesicular Content and Exocytotic Release with Two Electrodes in and at a Single Cell**
Chaoyi Gu, Andrew G. Ewing
Accepted for publication in *Chemical Science*, 2021, DOI: 10.1039/D1SC01190A

Participated in designing the experiments with A. E. Performed the experiments and analyzed the data. Interpreted the data and discussed the results with A.E. Outlined and wrote the first draft of the manuscript. Contributed to editing the manuscript with A. E.

† These authors contributed equally to the work.

Related papers not included in the thesis

Using Single-Cell Amperometry and Intracellular Vesicle Impact Electrochemical Cytometry to Shed Light on the Biphasic Effects of Lidocaine on Exocytosis
Daixin Ye, Chaoyi Gu, Andrew G. Ewing. *ACS Chem. Neurosci.* 2018, 9, 2941-2947

Mass Spectrometry Imaging Shows Modafinil, a Student Study Drug, Changes the Lipid Composition of the Fly Brain
Mai H. Philipsen†, Elias Ranjbari†, Chaoyi Gu, Andrew G. Ewing. Submitted

Omega-3 and 6 Fatty Acids Alter Membrane Lipid Composition and Vesicle Size to Regulate Exocytotic Release Rate and Transmitter Storage
Chaoyi Gu, Mai H. Philipsen, Andrew G. Ewing. Manuscript in preparation

A Multimodal Electrochemical Approach to Measure the Effect of Zinc on Vesicular Content and Exocytosis of Chemical Release on Oxygen-Glucose Deprivation and Reperfusion
Ying Wang, Chaoyi Gu, Andrew G. Ewing. Manuscript in preparation

The Effects of Tamoxifen and Cisplatin on the Lipid Composition of Fly Brains
Mai H. Philipsen†, Elias Ranjbari†, Chaoyi Gu, Andrew G. Ewing. Manuscript in preparation

Glutamate Receptors Regulate Exocytosis and Fraction of Release in Adrenal Chromaffin Cells
Ying Wang, Chaoyi Gu, Mohaddeseh Aref, Pieter Oomen, Amir Hatamie, Andrew G. Ewing. Manuscript in preparation

Electrochemistry at and in Single Cells
Alex S. Lima, Chaoyi Gu, Keke Hu, Andrew G. Ewing. Chapter 7 in ¨Electrochemistry for Bioanalysis¨, Elsevier Inc., 2021

† These authors contributed equally to the work.

Table of contents

Abbreviations

CNS	Central nervous system
PNS	Peripheral nervous system
ER	Endoplasmic reticulum
GABA	γ-aminobutyric acid
DOPA	Dihydroxyphenylalanine
TH	Tyrosine hydroxylase
VMAT	Vesicular monoamine transporter
DAT	Dopamine transporter
MAO	Monoamine oxidase
COMT	Catechol O-methyltransferase
PNMT	Phenylethanolamine-N-methyltransferase
NET	Norepinephrine transporter
TPH	Tryptophan-5-hydroxylase
SERT	Serotonin transporter
PMAT	Plasma membrane monoamine transporter
SSVs	Small synaptic vesicles
LDCVs	Large dense-core vesicles
VNC	Ventral nerve cord
TEM	Transmission electron microscopy
ATP	Adenosine triphosphate
V-ATPase	Vacuolar-type ATPase
VNUT	Vesicular nucleotide transporter
L-DOPA	L-3,4-dihydroxyphenylalanine
SNARE	Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor
v-SNAREs	Vesicle SNAREs
t-SNAREs	Target SNAREs
NSF	N-ethylmaleimide-sensitive fusion protein

SNAP	Soluble NSF-attachment protein
NMJ	Neuromuscular junctions
PC12	Pheochromocytoma
CaMKII	Calcium-calmodulin dependent protein kinase II
PKC	Protein kinase C
MPH	Methylphenidate
PC	Phosphatidylcholine
PE	Phosphatidylethanolamine
MSI	Mass spectrometry imaging
NGF	Nerve growth factor
UAS	Upstream activation sequence
IVIEC	Intracellular vesicle impact electrochemical cytometry
ROS/RNS	Reactive oxygen and nitrogen species
SCA	Single cell amperometry
VIEC	Vesicle impact electrochemical cytometry
DMSO	Dimethyl sulfoxide
IPE	Intracellular patch electrochemistry
CV	Cyclic voltammetry
FSCV	Fast scan cyclic voltammetry
FFNs	Fluorescent false neurotransmitters
GFP	Green fluorescent protein
TIRF microscopy	Total internal reflection fluorescence microscopy
STED microscopy	Stimulated emission depletion microscopy
PI	Phosphatidylinositol
CoA	Coenzyme A
MALDI	Matrix-assisted laser desorption ionization
SIMS	Secondary ion mass spectrometry
ToF-SIMS	Time-of-flight SIMS

Chapter 1. The Basics of Cellular Communication

Cell-to-cell communication is essential and vital for the survival of multicellular organisms. In addition, it provides the fundamental for high-level brain functions including cognition, emotions, rational thinking, etc. Luckily, cellular communication is not an unchanged process. It constantly reforms to help the organisms to adapt to new and challenging environments. Therefore, it is understandable that dysfunction of cellular communication can lead to devastating outcomes such as neurological diseases and cognitive deficiency. This chapter aims to give an overview of the structure and function of the nervous system, and how signals are propagated inside the nervous system. The introduction of the four types of neurotransmitters being studied during this thesis work is also included.

1.1 An Overview of the Nervous System

The nervous system consists of two main parts, one is the central nervous system (referred to as the CNS) and the other is the peripheral nervous system (PNS). The CNS can be divided into two parts, the brain, which can be subdivided into several structures (brainstem, cerebellum, etc.), and the spinal cord. The major component of the PNS, on the other hand, is neurons and it includes the sensory neurons and the motor neurons. The sensory neurons function to pass on signals being received by the sensory receptors to the CNS. The sensory receptors are located either at the surface of or deeper into the body. The motor neurons can be either autonomic, regulating involuntary activities coming from cardiac muscle, smooth muscles, and glands, or somatic, controlling voluntary movements by linking the CNS components to the skeletal muscles. Additionally, the autonomic motor neurons make up a component of the enteric system and are responsible for the functionality of gastrointestinal system. The CNS and the PNS coordinate with each other within the nervous system to receive and process internal and external stimuli, and subsequently convey significant signals to control body movements as well as organ function.[1-3]

A variety of cell types exist in the nervous system and they can be categorized into two broad groups; nerve cells (or neurons) and glial cells (or glia). The typical morphology and structure of a nerve cell is shown in Figure 1. The three main features of most nerve cells in the vertebrate nervous system are the dendrites, the cell body and the axon. The dendrites serve as a receiving unit to receive synaptic signal inputs from other nerve cells. The complexity of the dendritic branch determines the level of signal inputs a

specific nerve cell can take in. The cell body of a nerve cell contains organelles that can be found in all other cells, such as mitochondria, endoplasmic reticulum (ER), Golgi apparatus, etc. It stores genomic DNA and synthesizes proteins that are required for the normal function of the entire nerve cell. The axon, which can be viewed as an extension of the cell body, functions to convey synaptic signals to neighboring nerve cells after the signals are being integrated. The axon typically branches at the end to form many terminal fibers. The length of an axon, which ranges from several millimeters to a couple of meters, is mainly determined by the function of the nerve cell.

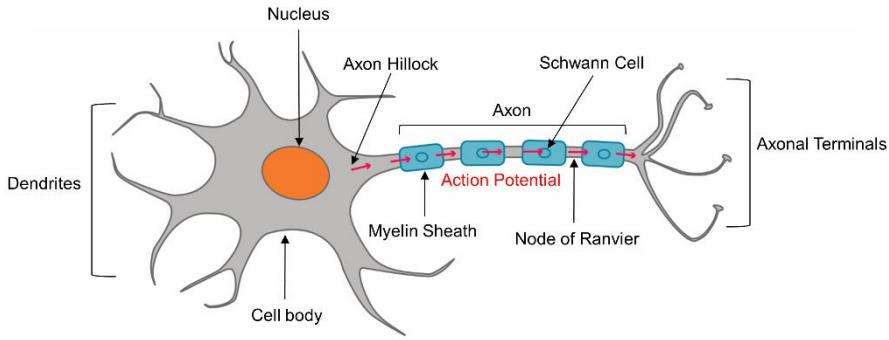

Figure 1. Illustration of the structure of a nerve cell. The three main features of a nerve cell are the dendrites, the cell body, and the axon. The axon is insulated by myelin sheath, which is formed by Schwann cells, and the myelin sheath is interrupted by nodes of Ranvier. An action potential is generated at the axon hillock and propagated along the axon to reach the axonal terminals.

Despite the important role of nerve cells in signal transduction, the amount of nerve cells in the vertebrate CNS is much less (approximately 2-10 times) compared to the other main cell type, the glial cells. Differing from the function of the nerve cells, glial cells are not directly involved in signal transduction. Instead, they are found surrounding the nerve cells and exert supportive roles to assist synaptic signaling. The major functions of glial cells include, but are not limited to, offering a scaffold for the development of nerve cells, maintaining ideal ionic environment for signal transduction, assisting re-uptake and breakdown of released neurotransmitters, and insulating axons by forming myelin sheath to promote signal transmission along the axons.[1, 3]

1.2 Signal Transmission within the Nervous System

At the resting state, nerve cells maintain a negative electrical potential of around -40 to -90 mV, depending on which type of nerve cells, inside the

cell relative to the extracellular environment. This potential is termed the resting membrane potential and is determined by unequal distributions of different ions inside versus outside the membrane, as well as the abilities of different ions to travel across the membrane.[4, 5] In general, K^+ ions are more concentrated inside the cell than outside, whereas Na^+ and Cl^- ions have higher concentrations outside.[6] Additionally, some amino acids and proteins, which are negatively charged, exist intracellularly and cannot move freely across the membrane. The ability of a certain ion to travel across the membrane depends on the number of its ion channels and whether these channels are open or not at a certain stage. The opening status of the K^+ channels in resting nerve cells leads to a higher permeability towards K^+ compared to the other ions. Therefore, the resting membrane potential is nearer the equilibrium potential of K^+ ions, which is around -75 mV. However, due to the concentration gradient across the membrane, some ions might leak out of or sneak into the membrane along their concentration gradients which could eventually disrupt the resting membrane potential. To prevent this, the Na^+-K^+ pump works to pump excess Na^+ ions out and meanwhile take in K^+ ions.[7, 8]

Upon receiving signals from adjacent nerve cells, the membrane potential can either be increased or decreased. The increase of membrane potential, once passing a certain threshold, generates an action potential at a region of the nerve cell called the axon hillock (Figure 1). The activities of voltage-gated Na^+ and K^+ channels play significant roles during this process.[4, 9, 10] As shown in Figure 2, at the resting state, the movement of Na^+ ions across the neuronal membrane is highly limited, while the movement of K^+ ions is much more favored, as described above. When a nerve cell is depolarized and the membrane potential reaches the threshold, the opening of voltage-gated Na^+ channels triggers the rapid influx of Na^+ ions (shown as the rising phase), which continuously drives the membrane potential towards the equilibrium potential of Na^+ ions (around +55 mV, shown as the overshoot phase). However, the opening of the Na^+ channels only lasts a few milliseconds, which is followed by the closing of the channels to decrease the permeability of Na^+ ions (shown as the falling phase). To further help to rapidly bring the membrane potential back to the resting state, voltage-gated K^+ channels open so that K^+ ions are able to move outwards (shown as the undershoot phase). These channels do not close until the membrane potential turns more negative than the resting membrane potential.

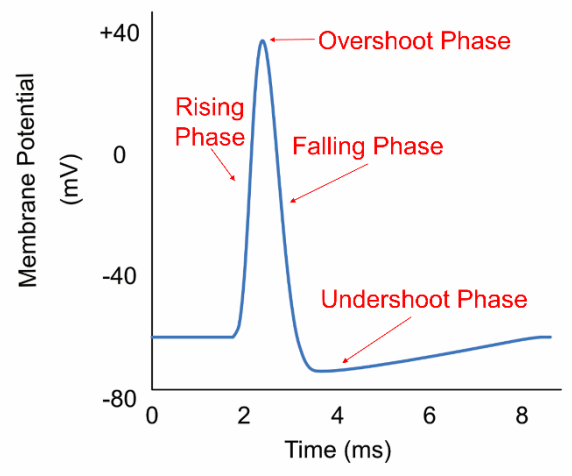

Figure 2. Illustration of the different phases of an action potential, including rising phase, overshoot phase, falling phase, and undershoot phase.

Since nerve cells are relatively poor conductors, the electrical signal that enters the cell during an action potential can leak out across the membrane, hindering signal transmission over a great distance. Insulating the axon with intervals of a myelin sheath is one way to facilitate the propagation of action potential within a cell. In the CNS, the myelin sheath is derived from the oligodendrocytes, one type of glial cells. In the PNS, the sheath is formed by Schwann cells. The myelin sheath that wraps the surface of the axon is regularly disrupted by gaps called nodes of Ranvier (Figure 1), where voltage-gated Na^+ channels can be found. These Na^+ channels function as enhancers of the action potential and the existence of the nodes of Ranvier ensures continuous generation of the action potential along the axon. The action potential ¨jumps¨ from one node to another and signal transmission for a long distance can be achieved.[1]

Synaptic transmission enables the communication between nerve cells. The cell that conveys the synaptic signal is referred to as the presynaptic cell, and the cell that receives the signal is the postsynaptic cell. The structure formed between the pre- and postsynaptic cells to transmit synaptic signals is termed the synapse, and synapses can be either electrical or chemical. Electrical synapses are useful for the passage of simple and fast electrical signals.[11] By linking the cytoplasm of the pre- and postsynaptic cells via specialized channels called gap-junction channels, electrical signals from the presynaptic cells can directly flow through these channels into the postsynaptic cells to induce depolarization.[12] In order for this to occur, the

distance between the pre- and postsynaptic cells needs to be extra small, down to a few nanometers. Since the agent of transmission at electrical synapses is electrical current, the direction of transmission is typically bidirectional.

Compared to the electrical synapses, the speed of transmission is slower at chemical synapses and synaptic delay of a few milliseconds is typically observed. The membranes of the pre- and postsynaptic cells at chemical synapses are not connected. Instead, a distance of around 20-40 nm is present between the two membranes and this space is called the synaptic cleft.[13, 14] Although lacking the speed of transmission that electrical synapses have, chemical synapses are dominant in the brain as they have the capability to transmit more complicated signals, leading to the generation of more complex behaviors.

At chemical synapses, signal transduction is accomplished via the release of signaling molecules, which are typically called neurotransmitters. When an action potential reaches the axonal terminal of the presynaptic cell, it induces a change of the presynaptic membrane potential and thus initiates the opening of the voltage-gated Ca^{2+} channels. The rapid influx of Ca^{2+} ions allows secretory vesicles to fuse with the presynaptic membrane to trigger neurotransmitter release.[15-17] This process is called exocytosis and will be introduced in detail in Chapter 2. The released neurotransmitters then diffuse across the synaptic cleft to reach and bind to certain receptors on the postsynaptic cell membrane. This binding can regulate the status of ion channels on the postsynaptic cell and induce ion flows across the membrane. Based on the properties of the postsynaptic receptors, the ion flows can either increase the postsynaptic membrane potential, an excitatory effect leading to the firing of an action potential, or decrease the membrane potential, an inhibitory effect which decreases the possibility of generating an action potential. Due to the fact that neurotransmitter, as the agent of chemical transmission, can only be released from the presynaptic side and act on the postsynaptic side, chemical transmission is usually in one direction, which is different from electrical transmission. Another important aspect regarding chemical synapses is the amplifying effect, meaning that a single secretory vesicle is capable of releasing thousands of chemical transmitters which can regulate thousands of ion channels in the target cell membrane and, thus, induce more complex behaviors. The change introduced to the target cell can last from a couple of seconds to several minutes or even longer.

A concept being studied in this thesis work is plasticity. Synaptic plasticity plays important roles in the processes of learning and memory formation.[18, 19] It refers to the change of synaptic transmission strength in

response to neural activity, and the duration ranges from the time scale of milliseconds to life-long.[20] Short-term plasticity mainly occurs at the presynaptic side and is usually shown as an alteration of neurotransmitter release.[21] This change can be either an enhancement, during which more neurotransmitters are released, or a reduction called synaptic depression. Synaptic depression leads to a decrease in neurotransmitter release, and the reason has been suggested to be the loss of number of ready-to-release vesicles induced by repetitive or high-strength neuronal firing. A change of synaptic transmission strength which lasts longer than 30 min is considered to be long-term.[22-25] Modification of certain proteins and regulation of neurotransmitter receptors on the postsynaptic side contribute to the initiation of long-term plasticity. In addition to that, alteration of gene expression levels during long-term plasticity is essential for longer or even permanent changes of synaptic transmission strength, which eventually modifies brain function.

1.3 Types of Biogenic Amine Neurotransmitters

Generally, a molecule that is stored inside the presynaptic nerve cell, can be released upon depolarization, and has specific receptors reside on the postsynaptic cell membrane is defined as a neurotransmitter. However, as more molecules which do not meet all the criteria have been discovered as neurotransmitters, this definition is limited. One main factor contributing to the complexity of chemical transmission is the diversity of neurotransmitters. More than 100 neurotransmitters have been identified and they are generally divided into two broad groups: small-molecule neurotransmitters and neuropeptides.[1] Small-molecule neurotransmitters can be further categorized into four smaller groups: acetylcholine, amino acids (glutamate, aspartate, γ-aminobutyric acid (GABA), and glycine), biogenic amines (dopamine, norepinephrine, epinephrine, serotonin, and histamine), and purines. Neuropeptides are relatively larger than the small-molecule neurotransmitters and consist of multiple amino acids.[26] Based on the postsynaptic effect, neurotransmitters can be excitatory or inhibitory. The main excitatory transmitter in the CNS is glutamate, while GABA is employed by most inhibitory synapses. The main focus of this thesis work is on dopamine. Norepinephrine, epinephrine, and serotonin have also been studied. The structures of the four neurotransmitters are shown in Figure 3. Several aspects including synthesis, action, catabolism, and functions of these transmitters will be introduced in the following text.

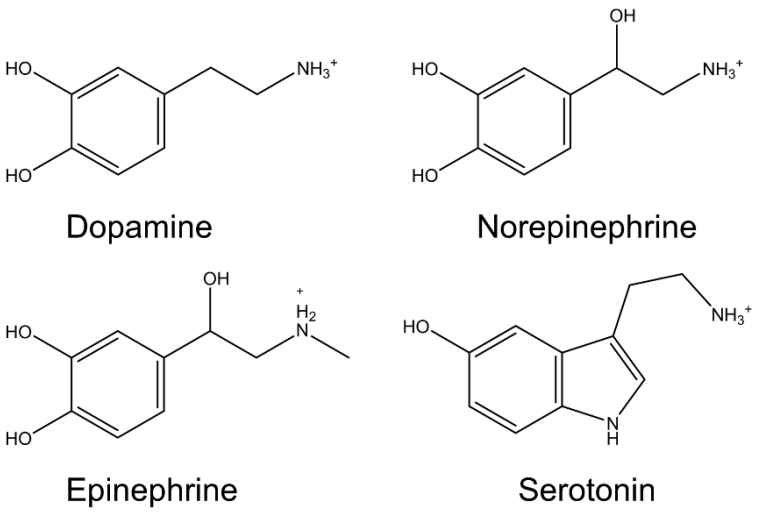

Figure 3. Chemical structures of the four neurotransmitters studied in this thesis work, including dopamine, norepinephrine, epinephrine, and serotonin.

1.3.1 Dopamine

Dopamine, besides being the precursor of norepinephrine and epinephrine, was found to be a neurotransmitter itself by Arvid Carlsson and coworkers in 1958. Due to this discovery and the study regarding the role of dopamine in the nervous system, Carlsson was awarded the Nobel Prize in the year 2000.[27-29] Dopamine may not be the most abundant neurotransmitter in the CNS, but the role of dopamine in coordinating body movements is highly important. The motor dysfunction phenomenon observed in patients with Parkinson's disease is caused by the degeneration of dopamine neurons.[30] Moreover, the significance of dopamine in aging, reward, drug addiction, and attention has also been demonstrated.[7, 31-34]

The biosynthesis of catecholamines (dopamine, norepinephrine, and epinephrine) all begin with the same precursor, the amino acid tyrosine. Dopamine is synthesized mainly inside the cytoplasm, during which tyrosine is first converted to dihydroxyphenylalanine (DOPA) with the presence of two cofactors, oxygen and tetrahydrobiopterin. The reaction is catalyzed by the enzyme tyrosine hydroxylase (TH), which is the rate-limiting enzyme for the synthesis of the three catecholamines. In the next step, the enzyme DOPA decarboxylase catalyzes the reaction to convert DOPA to dopamine. To be ready for chemical release, the newly synthesized dopamine is packaged into secretory vesicles through a transporter on the vesicle membrane called

vesicular monoamine transporter (VMAT).[35] When dopamine is released, it can interact with specific dopamine receptors, which subsequently regulates the activity of adenylyl cyclase to affect the second messenger system and intracellular signaling. The dopamine receptors are G-protein-coupled receptors and have five subtypes, including D_1 to D_5.[36] The clearance of the released dopamine is achieved by the reuptake process. Both the nerve cells and the supporting glial cells possess dopamine transporters (DAT) on their membranes. Lastly, the degradation of dopamine relies on two main enzymes, monoamine oxidase (MAO) and catechol O-methyltransferase (COMT).[37]

1.3.2 Norepinephrine and Epinephrine

The typical release sites of norepinephrine include nerve cells located in the locus coeruleus in the CNS, sympathetic ganglion cells located outside the brain, and adrenal glands. Epinephrine, on the other hand, is less abundant than the other two catecholamines and is mainly found in the chromaffin cells of the adrenal medulla, as well as some epinephrine-containing neurons in the CNS. The norepinephrine level in the brain is related to sleep and wakefulness.[38] In the body, norepinephrine and epinephrine are normally responsible for regulating heart rate and blood pressure. More importantly, under stressful or dangerous conditions, the levels of norepinephrine and epinephrine rise significantly, which is described as the fight-or-flight response.[39]

Unlike the synthesis of dopamine, the synthesis of norepinephrine takes place predominantly inside secretory vesicles and uses dopamine as the direct precursor. The conversion from dopamine to norepinephrine is catalyzed by the enzyme dopamine-β-hydroxylase with the presence of two cofactors, oxygen and ascorbic acid. Norepinephrine can be further converted to epinephrine, which is catalyzed by the enzyme phenylethanolamine-N-methyltransferase (PNMT) and uses S-adenosyl methionine as a cofactor.[40] However, as PNMT is present in the cytoplasm (mainly in adrenal chromaffin cells), the synthesis of epinephrine requires norepinephrine to be moved out of secretory vesicles. After being synthesized, the loadings of norepinephrine and epinephrine into vesicles are both achieved by the VMAT, the same vesicular transporter utilized by dopamine. Upon being released, both types of neurotransmitters can interact with the same group of receptors called the adrenergic receptors. These are G-protein-coupled receptors and have two main subtypes, α and β. Norepinephrine transporter (NET) is responsible for the reuptake of norepinephrine as well as epinephrine from the synaptic cleft back into the nerve cell, and the breakdown of these two neurotransmitters are largely accomplished by MAO and COMT.

1.3.3 Serotonin

The name serotonin originated from its function of regulating vascular tone when being discovered in the blood serum.[41] A few years later, serotonin was found to be present in the CNS.[42] It is a monoamine neurotransmitter and has multiple storage and release sites within the body.[43] A majority of serotonin is released by the enterochromaffin cells inside the gastrointestinal tract to control the movement of the intestines. The released serotonin can then enter the blood and subsequently be taken up by blood platelets. The blood platelets serve as a storage place for serotonin and utilize serotonin to adjust vascular tone. In the CNS, a small amount of serotonin is produced by the serotonergic neurons in the brainstem. However, the functions of serotonin in the CNS are complicated and nonnegligible, such as regulating sleep and wakefulness, and influencing mood, emotion and appetite.[44] Additionally, the involvement of serotonin in certain mental disorders, including depression and anxiety, has been suggested and many medications aiming to increase the extracellular concentration of serotonin for a longer period of time have been developed to ease the symptoms.[45]

The precursor of serotonin synthesis is the essential amino acid tryptophan, which is different from the precursor for catecholamines. The synthesis of serotonin involves two steps. Tryptophan is hydroxylated to 5-hydroxytryptophan in the first step, catalyzed by the enzyme tryptophan-5-hydroxylase (TPH), which is the rate-limiting enzyme for serotonin synthesis. There are two forms of TPH, TPH1 and TPH2, and only TPH2 is found to be present in nerve cells.[46] The second biosynthetic step for serotonin is the conversion from 5-hydroxytryptophan to serotonin. The reaction is catalyzed by the enzyme aromatic L-amino acid decarboxylase with pyridoxal phosphate as a cofactor. Upon synthesis, serotonin is first stored in secretory vesicles via VMAT and then later released into synaptic cleft to affect postsynaptic receptors. There are a variety of serotonin receptors, ranging from 5-HT_1 to 5-HT_7, and some subtypes can be divided into even smaller groups. Most of the serotonin receptors are G-protein-coupled receptors except one subtype, 5-HT_3, which is a ligand-gated ion channel.[47] After the action of serotonin, reuptake occurs via a serotonin transporter called SERT, which specifically pumps serotonin back into the nerve terminals. Therefore, a number of antidepressant drugs target SERT to elevate extracellular serotonin level. Recently, another transporter called the plasma membrane monoamine transporter (PMAT) has been identified which also serves to remove serotonin from the synaptic cleft.[48] The main pathway to degrade serotonin is mediated by MAO, the same enzyme that breaks down other

monoamines, thus making MAO an alternative target for treating depression and anxiety.

Although the four monoamine transmitters described above only account for a small part of the total neurotransmitters, the release of them via exocytosis has high impacts on a variety of body functions and complex behaviors. The next chapter will introduce in detail about this release process and its regulatory mechanisms.

Chapter 2. Exocytosis and Its Regulation

At chemical synapses, neuronal communication occurs via a vital process called exocytosis, during which secretory vesicles fuse with the cell membrane to discharge the stored neurotransmitters into the synaptic cleft. Due to its importance, exocytosis is tightly regulated. In addition, the process and regulatory machinery of exocytosis are highly conserved among multicellular organisms. There are several steps involved in exocytosis, including docking, priming, and fusion. This chapter focuses on an introduction regarding the structure of secretory vesicles, the pathway of exocytosis and involved proteins, as well as how exocytosis is regulated.

2.1 Types of Secretory Vesicles and Their Structures

The cellular organelles that serve as functional units to store and release neurotransmitters, as well as other signaling molecules, during chemical communication are called secretory vesicles. Based on the size and whether a protein core is present or not, there are mainly two kinds of secretory vesicles, small synaptic vesicles (SSVs) and large dense-core vesicles (LDCVs). Figure 4A shows the presence of SSVs and LDCVs in a *Drosophila* ventral nerve cord (VNC). SSVs have a diameter of around 50 nm and due to the small size, they enclose only a limited amount of internal proteins.[49, 50] Under transmission electron microscopy (TEM), these vesicles appear to be electron-lucent, which underlies the main structural difference from LDCVs. Small-molecule neurotransmitters including acetylcholine, GABA, and catecholamines are typically stored in SSVs and released when an action potential reaches the nerve terminal. LDCVs, on the other hand, are bigger in terms of size. The diameter of LDCVs is between 100-300 nm and a protein core is present. Due to the sample preparation process, the protein core is heavily stained and appears to be electron-dense under TEM. Surrounding the protein core is an electron-lucent region which is commonly called the halo. Similar to SSVs, LDCVs can store some small-molecule neurotransmitters such as catecholamines, serotonin, and histamine. In addition, neuropeptides are co-packaged inside LDCVs and can be co-released with other neurotransmitters in accordance with different patterns of neuronal activity.[51]

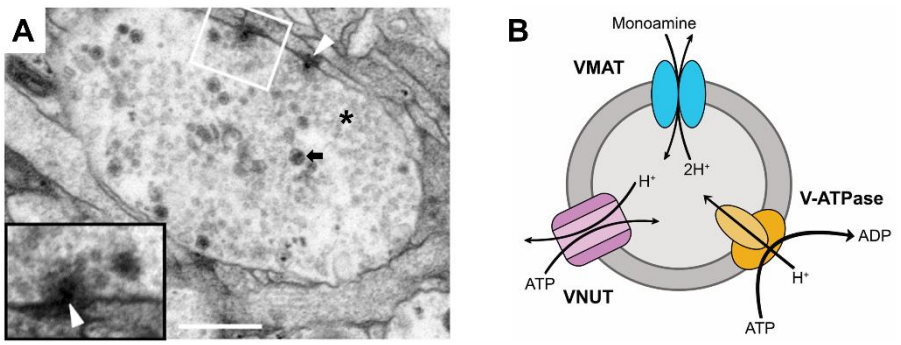

Figure 4. A. A TEM image of a neurite in a *Drosophila* VNC which contains both SSVs and LDCVs. The inset is enlarged from the white box. The triangle shows a synapse, small black arrow shows a LDCV, and asterisk shows a cluster of SSVs. Scale bar is 500 nm. Reproduced with permission from ref 52. B. A schematic showing the structure of a secretory vesicle with the presence of different transporter proteins, including V-ATPase, VMAT, and VNUT.

Both types of secretory vesicles are originally synthesized in the ER and the Golgi apparatus inside the cell body, during which the protein dense-core and neuropeptides are also synthesized and loaded into the LDCVs. With the help of motor proteins such as kinesin, the vesicles are then transported actively along the microtubules to the nerve terminals, where synthesis and subsequent loading of small-molecule neurotransmitters take place. The ability to synthesize small-molecule neurotransmitters near the release sites enables rapid replenishment and reloading of vesicles in the circumstance of high-frequency firing. However, this is not the case for neuropeptides, which can only be synthesized in the cell body. Moreover, the amount of neuropeptides released by LDCVs is relatively lower compared to other neurotransmitters, but the action lasts much longer.[2] In the CNS, SSVs are mainly located as clusters at presynaptic terminals, while LDCVs are also found in places like cell bodies and varicosities.[53, 54] Outside the CNS, LDCVs are present in neuromuscular junctions and neuroendocrine cells, such as chromaffin cells in the adrenal glands, and enteroendocrine cells in the gastrointestinal tract and pancreas.[55-57]

In LDCVs, the dense-core mainly consists of a group of acidic proteins known as the chromogranins, which includes chromogranin A, B, and C (also called secretogranin II).[58] Strong binding abilities of chromogranins towards Ca^{2+}, adenosine triphosphate (ATP), and some neurotransmitters including catecholamines have been suggested.[59, 60] Secretory vesicles are specialized in terms of concentrating neurotransmitters and in the case of LDCVs, an average concentration of 0.7-0.9 M neurotransmitters can be

accumulated inside.[61] Therefore, the presence of chromogranins is considered to tightly bind and aggregate a fraction of neurotransmitters in order to reduce the osmotic pressure inside the vesicles, which would otherwise cause the vesicles to burst.[62] Another important aspect regarding the existence of the protein dense-core is to regulate the amount and time course of neurotransmitter release, which has been demonstrated via knock-out or overexpression of specific chromogranins. Knock-out of chromogranin A and/or B clearly reduces the amount of release and accelerates the release dynamics, whereas overexpression yields opposite effects.[63-65] Additionally, as neurotransmitters that bind to the chromogranins cannot move freely, while the rest of the neurotransmitters which are stored in the halo region of the LDCVs have less-hindered diffusion, differential release dynamics from these two compartments have been suggested.[66] Besides binding to the protein dense-core, catecholamines can also have interactions with ATP to form weak complexes.[67, 68]

Among all the proteins of LDCVs, intra-vesicular proteins account for roughly 80% and the rest are mostly membrane-bounded proteins, including transporter proteins and trafficking proteins. Trafficking proteins will be described in detail in section 2.2. Three vesicular transporter proteins, namely vacuolar-type ATPase (V-ATPase), VMAT, and vesicular nucleotide transporter (VNUT), play crucial roles in maintaining the regular function of vesicles. The structure of a vesicle with the three transporter proteins is depicted in Figure 4B. V-ATPase is a highly conserved enzyme found in eukaryotic cell membranes.[69] The function of V-ATPase is to generate a proton gradient across the vesicle membrane by pumping protons towards the inside of vesicles, utilizing the energy harvested from breaking down ATP.[70] The accumulation of protons thus creates an acidic environment within vesicles resulting in a pH of around 5.5, in comparison to the neutral pH in the cytoplasm. This relatively low pH is necessary to maintain the aggregated form and optimal binding capacity of chromogranins. Moreover, the other two transporters, VMAT and VNUT, use the proton gradient to import monoamines and ATP, respectively, from the cytosol. It has been shown that VMAT pumps out two protons in exchange for one monoamine accumulating inside the vesicle. The process begins via intra-vesicular binding between a proton and the transporter, which induces a change of the transporter structure and allows one cytosolic monoamine to attach to the active transport site. The subsequent binding of the other proton leads to a further structural change and thus, the monoamine can be released into the vesicle and meanwhile the two protons are expelled.[71, 72] Reserpine, a drug used to lower blood pressure, has been proposed to inhibit VMAT by acting on the transporter after the binding of the first proton.[73] Treating neuroendocrine cells with reserpine leads to

decreases of vesicle volume and transmitter level mediated by VMAT. In contrast, L-3,4-dihydroxyphenylalanine (L-DOPA), the precursor of catecholamines, increases both by promoting vesicle loading of excess neurotransmitters via VMAT.[74] To date, two types of VMAT have been identified, including VMAT1 which is expressed exclusively in neuroendocrine cells, and VMAT2 which is located primarily in the CNS.[72] VNUT functions to pump protons out of vesicles to exchange for ATP.[75] The presence of intra-vesicular ATP is important when considering its interaction with chromogranins as well as catecholamines. As a neurotransmitter itself, the storage and release of ATP is essential during purinergic signaling.[76]

2.2 The Exocytotic Pathway and Related Proteins

The life cycle of a secretory vesicle in a nerve terminal is depicted in Figure 5A. As introduced previously, vesicles are synthesized in the neuronal cell body and transported to the nerve terminal where neurotransmitters are loaded. A fraction of these loaded vesicles constitutes a vesicle pool called the reserve pool, within which vesicles do not release in response to normal stimulation.[77] Studies have suggested that a membrane protein called synapsin functions to cross-link the vesicles within the reserve pool.[78] In addition, synapsin also binds to actin filaments in the cytoskeleton and thus prevents vesicles from moving freely.

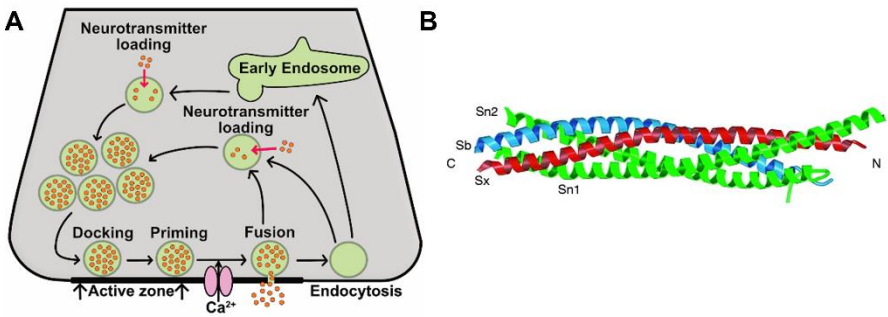

Figure 5. A. Classical illustration of the life cycle of a secretory vesicle in a nerve terminal. The vesicle is first loaded with neurotransmitters, and then goes through a series of processes including docking, priming, and fusion before releasing the neurotransmitters, and in the end is recycled via endocytosis. Adapted from ref 79. B. Structure of the zippered SNARE complex, consisting of synaptobrevin (blue), syntaxin (red), and two SNAP-25 (green) proteins. Reproduced with permission from ref 80.

High-frequency stimulation or a strong depletion of other vesicle pools is thought to be able to phosphorylate synapsin, which liberates vesicles

inside the reserve pool and allows them to migrate and attach closely to release sites on the cell membrane, a process typically referred to as docking. However, the mechanism underlying docking is not yet well understood. Although a variety of proteins have been identified to assist vesicle docking, absence of these proteins does not terminate this process.[81, 82] The docked vesicles are considered to make up another vesicle pool called the readily releasable pool and when stimulation occurs, these vesicles are the first ones to perform chemical signaling.[77] It should be noted that there is no difference when it comes to the natural properties of vesicles within different pools, even though they might have distinct functions and respond differently to stimulation.

After docking, vesicles must go through a process termed priming in order to be prepared for the subsequent fusion. Priming involves coordination of a great number of proteins and the most important ones are the soluble N-ethylmaleimide-sensitive-factor attachment protein receptor (SNARE) proteins. There are two types of SNAREs, vesicle SNARE (v-SNARE) which is synaptobrevin and is present on the vesicular membrane, and syntaxin and SNAP-25, target SNAREs (t-SNAREs) which are attached to the plasma membrane. Structures of all SNARE proteins share a common special sequence called the SNARE motif, which is made of 60-70 amino acid residues. Most SNARE proteins, including synaptobrevin and syntaxin, possess one SNARE motif in their structures, except SNAP-25 and its related proteins which have two SNARE motifs.[83] During priming, these four SNARE motifs assemble to generate a partially completed structure with four α-helixes, referred to as the SNARE complex. This is shown in Figure 5B.[80] This assembling process is also called SNARE zippering.[84] Since both vesicle and plasma membranes consist of negatively-charged lipid bilayers and have intrinsic stabilities, the formation of SNARE complex can help to overcome the energy barrier for fusion when the two membranes are brought close to each other.[85] Despite the importance of the SNARE proteins, assembling of the SNARE complex cannot take place without the help of several other proteins, such as SM proteins (e.g. Munc18) which have been suggested to bind syntaxin to promote the formation of the SNARE complex.[86, 87]

Once vesicles are primed, rapid influx of Ca^{2+} ions caused by the opening of a large number of Ca^{2+} channels on the plasma membrane triggers full zippering of the SNARE complex to open a fusion pore, through which neurotransmitters can be released into the extracellular space. The protein that senses and reacts to the entry of Ca^{2+} ions is suggested to be another vesicular membrane protein called synaptotagmin. In addition to binding Ca^{2+} ions, synaptotagmin is also capable of binding both SNAREs and phospholipids,

leading to the belief that insertion of synaptotagmin into the plasma membrane facilitates the full SNARE zippering.[88] Another set of proteins that is considered to be important for the action of Ca^{2+} ions are the RIMs. These proteins gather Ca^{2+} channels into release sites and thus enable rapid Ca^{2+} influx to then trigger neurotransmitter release.[89]

The SNARE complex must be disassembled after the fusion process so that vesicles can be recycled and meanwhile the t-SNAREs remain on the plasma membrane. Two proteins, N-ethylmaleimide-sensitive fusion protein (NSF) and soluble NSF-attachment protein (SNAP) are involved in the disassembling process and the energy required is provided by hydrolysis of ATP.[90] The recycling of exocytosed vesicles is achieved via a process termed endocytosis. Depending on whether clathrin is required, endocytosis can be divided into two distinct pathways, one fast and one slow. In chromaffin cells, fast endocytosis occurs within several seconds, whereas slow endocytosis takes several minutes to complete.[91] Through which pathway a vesicle is endocytosed is determined by the strength of stimulation, i.e. high-frequency stimulation can switch endocytosis from fast to slow mode.[79]

2.3 Different Modes and Regulatory Mechanisms of Exocytosis

The formation of a fusion pore during exocytosis allows the release of signaling molecules to the extracellular space, a key step in chemical signaling. It is well accepted that SNARE proteins are one of the important factors determining whether a fusion pore can be formed or not, but what occurs to the fusion pore afterwards is a controversial topic. The Ω-shaped structure of vesicle fusion was initially observed in the frog neuromuscular junction (NMJ) in the 1970s and was correlated with neurotransmitter release.[92] This observation supported the theory of quantal release discovered by Katz and coworkers, which is the earliest and most traditional view about exocytosis.[93] During quantal release, or also called full release, the fusion pore between the vesicle and plasma membranes continues to expand until the entire vesicle collapses and incorporates into the plasma membrane, leading to the discharge of all content including neurotransmitters and proteins if are present.

In the 1990s, the combination of capacitance measurements, which monitor changes in plasma membrane surface area in relation to vesicle fusion, and amperometric recording, which quantifies neurotransmitter release, gave rise to the discovery of transient fusion and the so-called kiss-and-run release.[94-96] During kiss-and-run release, the fusion pore has a

relatively small size and remains open only for a very short time before it closes again, allowing the escape of a small fraction of neurotransmitters.

In the past decade, thanks to the development of electrochemical techniques, a third mode of exocytosis called partial release, or subquantal release, has been proposed which in some ways falls between the previous two release modes.[97-103] During partial release, the initially formed, small-sized fusion pore continues to expand to a certain degree, but not completely, and then constricts back to a small size again and closes, during which a larger number of neurotransmitter molecules are released in comparison to the kiss-and-run mode. The theory of partial release and the dynamics of the fusion pore have been supported by an extensive amount of modeling work and studies employing advanced imaging techniques.[66, 104-106] Most studies have focused on exocytosis of LDCVs in neuroendocrine cell models, including chromaffin cells, and pheochromocytoma (PC12) cells, and it has been quantified that under normal conditions, a vesicle in these cells typically releases around 60% of its neurotransmitter content during exocytosis.[107] For both partial release and kiss-and-run release, closing of the fusion pore enables rapid and direct reuse of exocytosed vesicles. Illustrations of the three release modes described above are shown in Figure 6. In addition to these three modes, another one called flickering has also been discovered in some neurons, during which the fusion pore opens and closes rapidly and repetitively.[108, 109]

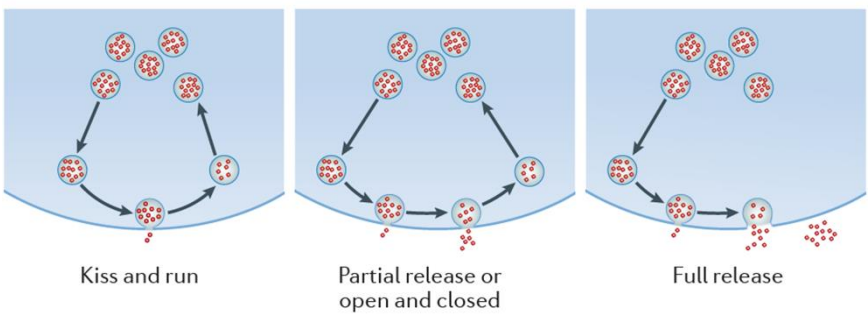

Figure 6. Three modes of exocytosis, namely kiss and run, partial release, and full release. All neurotransmitters are released during full release, whereas kiss and run and partial release only allow a fraction of the transmitter load to be released. Kiss and run releases a relatively low fraction of transmitters in comparison to partial release. Reproduced with permission from ref 100.

Regulation of exocytosis involves coordination of a large number of proteins as well as interactions between proteins and cell/vesicle membranes. Any step going incorrectly can cause unfavorable changes in neuronal communication which might further affect behaviors of organisms. As described in section 2.2, the SNARE proteins play key roles in the initiation of fusion pore opening. Neurotoxins including botulinum and tetanus damage structure of certain SNARE proteins to prevent vesicle fusion and neurotransmitter release, leading to fatal outcome.[110] In addition, stimulation pattern and intracellular Ca^{2+} level are also considered important factors in regulating exocytosis. It has been shown that increased frequency of action potentials (0.25-10 Hz) enhances both the number of release events and the average release amount per event in bovine chromaffin cells.[111] A similar trend was observed when a chromaffin cell was repetitively stimulated by a train of depolarizations.[112]

How repetitive stimulation alter exocytosis might vary among different cell types, but the involvement of Ca^{2+} underlies the common mechanism (**paper II**).[113]. The importance of Ca^{2+} in triggering exocytosis was demonstrated by injecting Ca^{2+} ions into the presynaptic nerve terminal. Without the presence of an action potential, the injected Ca^{2+} ions themselves are enough to induce exocytosis and the concentration of Ca^{2+} is proportional to the amount of vesicles that undergoes fusion process.[15, 111] In addition to interacting with exocytosis-related proteins, such as synaptotagmin and RIMs, to directly affect exocytosis, Ca^{2+} can also bind to some other proteins to induce phosphorylation, which subsequently alters exocytosis.[17] The activation of calcium-calmodulin dependent protein kinase II (CaMKII) via Ca^{2+}-binding is considered to phosphorylate synapsins, which allows vesicles from the reserve pool to supplement the readily releasable pool especially during high-frequency stimulation, and is therefore, necessary in the formation of activity-dependent plasticity.[114] Another protein, protein kinase C (PKC), can also be activated by Ca^{2+}. Phosphorylation of PKC speeds up vesicle translocation to increase the size of the readily releasable pool, a result similar to the action of CaMKII.[112] PKC can also affect exocytotic protein machinery and one example is that PKC-induced phosphorylation of Munc18 has been shown to change the dynamics of release from single vesicles.[115] Moreover, both Ca^{2+} and PKC are involved in regulating the actin cytoskeleton, which is important in maintaining cell shape and directing vesicle movements.[116]

In the theory of quantal release, vesicles undergoing exocytosis are more or less considered as a group or a unit. Release from individual vesicles seems not as important as long as the total release amount can elicit postsynaptic responses. In this case, alteration of exocytosis can be achieved

by changing the number of ready-to-release vesicles as well as manipulating vesicular neurotransmitter storage prior to exocytosis. Once these two factors are set, the total release amount is fixed since each vesicle releases all transmitter storage during quantal release. However, as exocytosis is a highly complexed process and is too critical to go unregulated, it is likely that more regulatory mechanisms are involved.

The discovery of partial release as a mode of exocytosis brings more possibilities to regulate exocytosis in a more dynamic manner. The amount of neurotransmitters originally stored inside vesicles, the amount released during exocytosis, and the dynamics of fusion pore (opening and closing) are all key players in terms of regulating exocytosis and can be altered differentially in response to pharmacological treatment (**paper I**) or cellular activity (**paper II**). L-DOPA treatment proportionally increases transmitter storage and release whereas reserpine gives opposite effects, both resulting in a nearly unchanged fraction of release (amount of release divided by amount of storage).[117] The actions of these two drugs on neurotransmission are mainly mediated through VMAT instead of through affecting the dynamics of release. Cocaine and methylphenidate (MPH) are known to inhibit dopamine uptake by blocking DAT. Cells treated with these drugs show decrease in vesicular content, as expected, but exhibit differential effects on the dynamics of fusion pore and the fraction of release which might underlie the mechanism behind cognition.[118] Additionally, several anticancer drugs and anesthetics are capable of inducing biphasic effects on exocytosis from relatively low to high concentrations by altering the dynamics of fusion pore.[119-121] Another important aspect regarding partial release is that regulation of exocytosis is more focused on the level of single vesicles instead of a group of vesicles. When considering 60% as the average release fraction from a cell,[107] some vesicles might release much less than 60% while some others might undergo full release, implicating possible heterogeneity of release machinery among vesicles. More importantly, an organism can utilize this heterogeneity to respond to rapid changes occurring in the surrounding environment.

Regulation of the fusion pore is of great importance in partial release, as the duration of the fusion pore determines how many signaling molecules can be released during exocytosis. In addition to the participation of SNARE proteins in the initiation of fusion pore, two other proteins, namely actin and dynamin, have been indicated to regulate opening and closing phases of the fusion pore. Using amperometric measurements, by inhibiting activities of dynamin and actin, the roles of dynamin in the expansion of fusion pore and actin in the constriction of the pore were suggested in PC12 cells, which was supported by another study carried out in mouse chromaffin cells.[122-124]

However, during endocytosis, dynamin is known to be involved in constricting the vesicle membrane to induce membrane scission, its similar function in exocytosis has also been proposed and examined.[105, 106, 125, 126] Although a consensus about which protein controls which phase of the fusion pore has not yet been reached, it appears that the interplay between actin and dynamin governs the dynamics and duration of fusion pore.

During exocytosis, merging between the vesicle and cell membrane is required for the formation of the fusion pore. To promote fusion, the phospholipid structure of the two membranes needs to be reorganized and during the subsequent expansion of the fusion pore, incorporation of extra lipids is required. This leads to the consideration that lipids can be essential factors in regulating exocytosis.[127-129] Effects of a variety of exogenous phospholipid species on exocytosis and the dynamics of fusion pore have been investigated in previous studies.[130, 131] It was found that the position of phospholipid incorporation matters greatly. Incorporating these lipids into the inner leaflet of the plasma membrane leads to different outcomes regarding the amount of release and the dynamics of the fusion pore in comparison to incorporating them into the outer membrane.[132] In addition, phospholipids with different shapes affect exocytosis differentially. Phosphatidylcholine (PC), which has a cylindrical shape, generates relatively low curvature when is present in the membrane and is therefore, less favored by the highly-curved fusion pore.[133] In contrast, conical-shaped phosphatidylethanolamine (PE) can produce high curvature and is more concentrated around the fusion pore. By combining amperometric measurements with mass spectrometry imaging (MSI), it has been suggested that a relatively small change in the plasma membrane lipid composition following phospholipid incubation is capable of altering exocytosis significantly.[131, 134] However, phospholipids are not the only components in the plasma membrane that are involved in regulating exocytosis, the roles of cholesterol and fatty acids regarding the formation of the SNARE complex also have significant impact.[135] Details about different phospholipids, fatty acids and cholesterol will be described in Chapter 5.

Due to the high complexity of exocytosis, a large amount of studies have been conducted focusing on different aspects related to exocytosis. The development of diverse biological model systems makes it possible to study exocytosis in a less complicated environment and to pinpoint specific questions, which will be the focus of the next chapter.

Chapter 3. Biological Systems Used as Models to Study Exocytosis

The ultimate goal of studying cellular communication is to understand the fundamental structure and function of the human brain. This is essential in revealing potential mechanisms underlying a variety of neurological diseases. However, many obstacles exist when it comes to studying the human brain. One of them is that the human brain is highly complicated, making it difficult to target specific questions. Therefore, it is of great demand to develop simplified systems. As the machinery of exocytosis is conserved among multicellular organisms, a great number of biological systems with less complicated structures have been used as models to study exocytosis. These include vertebrates like rats and mice, invertebrates such as *Drosophila*, as well as cells in culture, including endocrine cells. This chapter focuses mainly on introducing the three biological model systems used for this thesis work, which are PC12 cells, chromaffin cells, and *Drosophila melanogaster*. Background of these models and their applications in the research of exocytosis are addressed in detail. This chapter also includes a brief introduction of other biological models that are commonly used in the field of neuroscience.

3.1 PC12 Cells

One approach to create a simplified model system to study exocytosis is to use isolated cells, which includes primary cell cultures and cell lines. PC12 is one of the most commonly used cell lines and was first isolated from a tumor in the rat adrenal gland in 1976 by Lloyd Greene and coworkers.[136] It was observed that PC12 cells have a round or slightly oval shape and tend to form small clusters when growing them in culture. More importantly, this cell line can synthesize certain neurotransmitters and undergo Ca^{2+}-dependent exocytosis.[137] The main neurotransmitter synthesized and used in PC12 cells is dopamine, but a small amount of norepinephrine is sometimes also present. Unlike chromaffin cells, which also originate from the adrenal glands, PC12 cells do not contain PNMT, the enzyme required for the conversion of norepinephrine to epinephrine, and thus do not synthesize the transmitter epinephrine. PC12 cells possess LDCVs, within which dopamine and norepinephrine are stored, and these vesicles are typically smaller and store lower concentration of neurotransmitters than the ones in chromaffin cells.[136] Several studies have suggested, although it is not generally accepted, the possible existence of two classes of DCVs in PC12 cells with differences in size and neurotransmitter content.[138, 139] In addition to the release of certain

catecholamines, acetylcholine, which is stored inside small clear vesicles, can also be released from PC12 cells.[140] Figure 7 shows a TEM image of a PC12 cell with LDCVs inside.

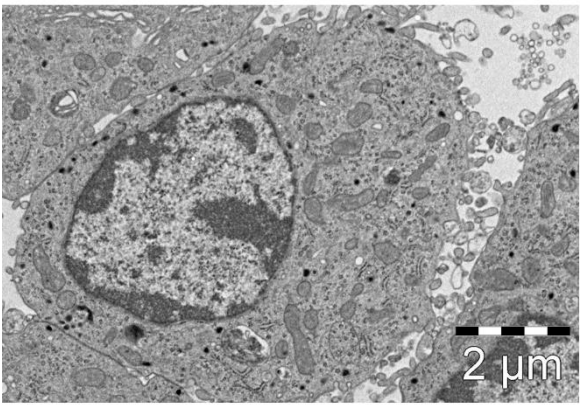

Figure 7. TEM image of a PC12 cell and LDCVs can be observed. Scale bar is 2 µm.

A few receptors have been found to be present on PC12 cells which are of interest for neuroscience research, including nicotinic receptors, muscarinic receptors and dopaminergic autoreceptors.[141, 142] Both nicotinic and muscarinic receptors can be activated to trigger exocytosis from PC12 cells, but via distinct pathways. The activation of the nicotinic receptor opens Na^+ channels to induce sufficient membrane depolarization, whereas the activation of muscarinic receptors acts on intracellular second messengers to trigger intracellular Ca^{2+} release.[141, 143] However, it has been shown that both pathways lead to significant delays of the initiation of exocytosis. In comparison, application of a high concentration K^+ solution induces direct membrane depolarization to rapidly trigger exocytosis from PC12 cells, making it the most frequently used stimulation method.[144] PC12 cells also express D_2 autoreceptors. The activation of these receptors offers feedback to affect neurotransmitter synthesis and therefore, regulate exocytosis.[145]

PC12 cells have the ability to be differentiated into a phenotype resembling sympathetic ganglion neurons. By exposing PC12 cells to nerve growth factor (NGF), cell bodies start to extend neurite-looking processes, which can be as long as 1000 µm, and varicosities are formed along these processes.[136] In differentiated PC12 cells, secretion typically takes place at varicosities or the ends of the extended processes instead of the cell bodies. It has been shown that the average amount of release from differentiated PC12 cells is similar to the amount measured in undifferentiated cells.[146] However,

the distribution of release tends to become narrower upon differentiation, suggesting secretion from a more homogeneous population of vesicles. In addition to NGF, PC12 cells can also be differentiated by glucocorticoid, which leads to enhanced excitability and faster exocytosis/endocytosis cycle.[147]

During the past several decades, PC12 cells have been a commonly used model to study the possible mechanism governing exocytosis.[137] The average amount of catecholamine release from vesicles in undifferentiated PC12 cells was first reported to be approximately 190 zmol, which is 114300 molecules.[148] By genetically manipulating specific genes or using transfection method to inhibit or overexpress certain proteins, a variety of PC12 cell types can be created aiming to understand the roles of specific proteins in exocytosis, such as SNARE proteins and chromogranins.[65, 149-151] Moreover, PC12 cells are largely used to investigate the action and mechanisms of many drugs, e.g. anticancer drugs, anesthetics, psychostimulants (**paper I**), etc., and mechanisms underlying certain diseases like hypoxia.[119-121, 152] As the PC12 cell line is derived from a tumor, it can be passaged infinitely which requires less lab work in comparison to culturing primary cells. However, a disadvantage of cancer cell lines is that characteristics of the cells often deviate from the originally isolated cells after multiple passaging cycles. Any deviations from the original cell culture protocol can and often do generate sub-clones of the cell line, which leads to difficulties in the ability to compare between different data sets. As long as this disadvantage is understood, PC12 cells can be a simple but highly useful model when it comes to studying a variety of research topics related to exocytosis as well as neuronal differentiation.

3.2 Chromaffin Cells

Another type of neuroendocrine cell that has been widely used in the research field of exocytosis is adrenal chromaffin cells. They are located mainly in the medulla of the adrenal glands and receive signal inputs from splanchnic nerve of the sympathetic nervous system. In mammals, when acetylcholine is released from the splanchnic nerve, nicotinic acetylcholine receptors can be activated which triggers the release of norepinephrine and epinephrine from chromaffin cells into systemic circulation. Chromaffin cells are commonly isolated from the adrenal glands and grown as primary cultures which survive for a couple of days. Typical morphology and structure of isolated bovine chromaffin cells are shown in Figure 8A and 8B. Based on which type of catecholamine is released, chromaffin cells can be either

adrenergic, secreting epinephrine, or noradrenergic, secreting norepinephrine. In addition to the chromaffin cells, another kind of secretory cell, cortical cell, is also frequently seen in the chromaffin cell cultures (Figure 8C). Cortical cells are isolated from the most inner layer of the adrenal cortex, which covers the adrenal medulla, and are responsible for secreting steroid hormones. There are a variety of chromaffin cells depending on which animal the adrenal glands come from, such as mouse, rat, and cow, and they exhibit distinct properties regarding expression of certain receptors and ion channels.[153-156]

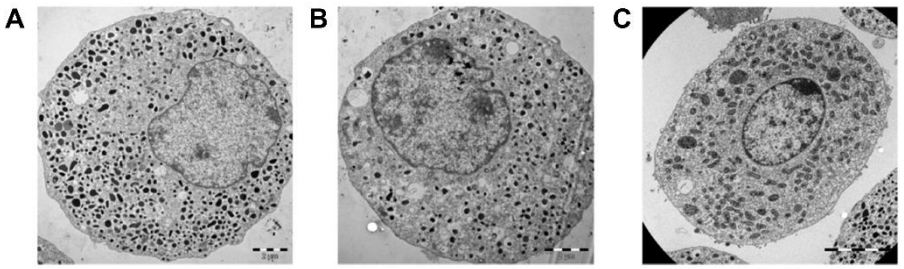

Figure 8. TEM images of three different secretory cells in the medulla and cortex of bovine adrenal glands, including A. adrenergic chromaffin cell (scale bar: 2 µm), B. noradrenergic chromaffin cell (scale bar: 2 µm), and C. cortex cell (scale bar: 5 µm). Reproduced with permission from ref 157.

In chromaffin cells, norepinephrine and epinephrine are synthesized, stored and released by LDCVs. These vesicles are larger than the ones in PC12 cells and can store a much higher concentration of catecholamines (up to 0.9 M).[61] Additionally, chromaffin cells can also store various neuropeptides as well as other types of large hormones like enkephalin. The co-release of catecholamines and larger hormones is important in the fight-or-flight response as well as maintaining normal body functions.[158] Similar to PC12 cells, chromaffin cells express both muscarinic and nicotinic receptors. The activation of either receptors by acetylcholine is capable of triggering the release of catecholamines.[159, 160] Chromaffin cells can also be chemically stimulated by high concentration of K^+ solution or interestingly, by Ba^{2+} ions.[161]

The advantages of using chromaffin cells as a model to study exocytosis include that they are more similar to healthy cells compared to PC12 cells. The larger vesicle size and amount of vesicular catecholamine content make it easier to measure vesicle fusion and quantify neurotransmitter release with electrophysiological and electrochemical techniques, respectively. However, as chromaffin cells are primary cultures, the life span

is typically a few days and the cells cannot be propagated. Therefore, relatively more lab work is required to maintain the culture of the cells. Another limitation of chromaffin cells comes from their animal origin, which not only might raise ethical issues, but also increases difficulties when it comes to interpreting and comparing data. Important factors such as quality of adrenal glands, batch-to-batch difference between glands, and on which day an experiment is performed after the cell isolation need to be taken into consideration when interpreting data.

3.3 *Drosophila Melanogaster*

Since its initial breeding by Charles W. Woodworth, *Drosophila melanogaster*, the fruit fly, has been widely used as a model organism in many research areas, especially in the fields of genetics and developmental biology. The initial motivation of using *Drosophila* as a model to study genetics comes from its short life cycle, high reproduction rate, and relatively simple genome (four pairs of chromosomes) compared to other animal models such as mice and rats.[162] The whole genome of *Drosophila* was sequenced in 2000 and a great level of similarity (up to 60%) has been found between *Drosophila* and humans despite the completely different appearances.[163] More importantly, it has been reported that more than 70% of genes which are involved in human diseases have a matching sequence in the *Drosophila* genome.[164] Additionally, approximately 60% of the human disease genes investigated by Rubin et al. have orthologs in *Drosophila*.[165] These similarities have led to the development of many disease-related *Drosophila* models aiming to study the mechanisms underlying a variety of human diseases, including neurodegenerative disorders, diabetes, and cancer.[166-168]

There are many advantages regarding using *Drosophila* as a model organism in scientific research. Cultivation of *Drosophila* in laboratories is a fairly simple process which does not require much cost and lab space. The lifespan of *Drosophila* is relatively short which reduces the amount of waiting time between rounds of experiments. Under 25 °C, it takes about 10 days for an embryo to develop into an adult fly.[162] In addition to the rapid life cycle, each female fly can lay up to 2000 eggs throughout the entire lifespan, offering a large number of offspring. The relatively simple genome and few genetic redundancies make it easy to genetically manipulate the *Drosophila* genome, enabling the generation of specific genotypes to answer certain research questions. Although having a less complicated nervous system, *Drosophila* show certain behaviors that are similar to mammals, such as circadian rhythm, pain, drug abuse, and learning and memory.[169, 170] Despite having all these

advantages, limitation of using *Drosophila* as a model for research need to be kept in mind. As an invertebrate organism, *Drosophila* is inherently different from humans and less complex in terms of high-level brain functions and social behaviors. Therefore, studies using *Drosophila* can reveal certain aspects of a research question, but the whole picture might be much larger.

The entire life cycle of *Drosophila* is depicted in Figure 9. It is made up of four different developmental stages: embryo, larva, pupa, and adult. The larval stage can be further divided into three sub-stages: first, second, and third instar. The lifespan of *Drosophila* is commonly around 50 days when maintained at a temperature under 25 °C. The growth process is affected by the environmental temperature with higher temperature speeding up the process while lower temperature slowing it down.[171] However, when the temperature reaches 30 °C, heat stress increases the time needed for fly development. To avoid confusion, all processes described below consider the environmental temperature to be 25 °C. The life of *Drosophila* begins with the embryo stage which normally lasts around one day, and then the first instar larva is hatched. The larva can grow for a total of four days, during which molting occurs twice enabling it to increase in size. Larvae spend most of the time eating and once reaching a certain time in the third instar stage, they crawl out of the food and pupate in a dry place. The third instar larva is commonly picked for research due to the relatively large size and the developed nervous system.[52] During the pupal stage, a hard shell is formed and the larva inside undergoes metamorphosis. This process typically requires four days and as a result, an adult fly emerges. It takes approximately ten hours for newly-emerged female flies to reach sexual maturation, and the virgin female flies are usually picked to cross with specific male flies to generate offspring with desired genotypes.

The third instar larva and the adult fly are two models that have been used to study exocytosis. The nervous system of a larva contains a CNS and a PNS. The CNS consists of two brain lobes (also called protocerebrum), which are made up of sensory neurons and interneurons, and a VNC which additionally contains some motor neurons. In the PNS, these motor neurons can form boutons, or also called varicosities, at NMJ to make contact with their target muscles inside the body wall in order to control movement of the larva. The nervous system of a larva is constantly under development from the first to the third instar stage and the estimated amount of neurons in the larval brain is 10000-15000.[172, 173] Figure 10A illustrates the structure of the larval CNS. Several neurotransmitters that exist in human brain are also found in the larval brain, including acetylcholine, glutamate, GABA, dopamine, serotonin, and tyramine.[174] The localization of dopamine and serotonin neurons in the

larval CNS are shown in Figure 10B. In addition, one special type of neurotransmitter, octopamine, is also present in larvae.[175] Octopamine is commonly found in invertebrates and has functions that are similar to the neurotransmitter norepinephrine in mammalian brains.

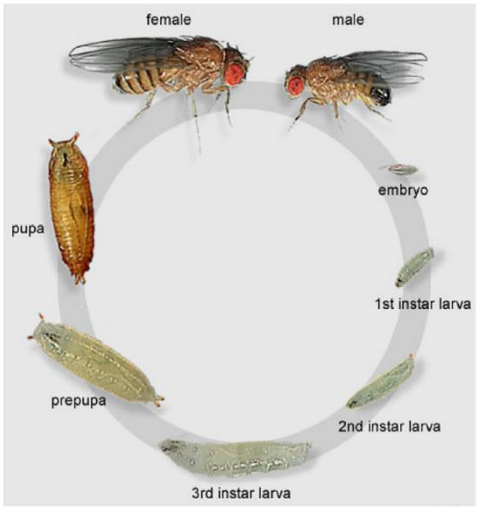

Figure 9. Life cycle of *Drosophila melanogaster*. The cycle consists of several developmental stages including embryo, larva, pupa, and adult. The developmental time taken from an embryo to an adult fly is around 10 days. Reproduced with permission from ref 176.

The adult fly brain, on the other hand, is more complicated and contains approximately 10 to 20 times more neurons compared to the larval brain.[173] Moreover, behavioral and cognitive studies are prevalently done in adult flies due to the reason that the adult brain has highly organized and specialized regions to guide complex behaviors and develop memories.[177, 178] As depicted in Figure 10C, the adult brain can be divided into several distinct regions, namely, mushroom bodies, central complex, antennal lobe, medulla, and lobula. The mushroom bodies and central complex are important parts in the central brain. The mushroom bodies are known as the center for learning and memory, not only for olfactory processing, but also for visual and other sensory functions.[179] The central complex functions to link different parts in the central brain, and is responsible for integrating input information and directing behaviors. Most types of neurotransmitters present in the larval brain can be found in the adult brain.[174, 180] In addition, histamine also exists in the adult brain and is involved in the visual system.[181]

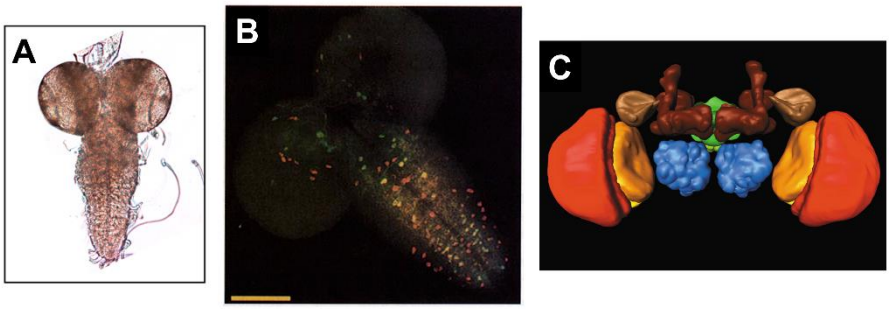

Figure 10. Nervous systems of larval and adult *Drosophila*. A. Structure of the CNS of the third instar larva, including two brain lobes and the VNC. Reproduced with permission from ref 182. B. Localization of dopamine (red) and serotonin neurons (green and yellow) in the larval CNS. Reproduced with permission from ref 183. C. Anatomical regions of the adult fly brain with the mushroom bodies shown in dark brown, central complex in green, antennal lobe in blue, medulla in red, and lobula in orange. Reproduced with permission from ref 184.

A large number of fly genotypes has been created for specific research purposes and a high fraction of them are obtained via genetic manipulation, e.g. overexpression, knockout or knockdown, mutation, and insertion of certain genes. One simple and widely applied method to generate specific fly genotypes is by using the Gal4-UAS system, a powerful gene expression technique. The Gal4 system was initially found in yeast and has since then been broadly applied for the activation of specific genes in mammalian cells and other organisms, including *Drosophila*.[185-187] The Gal4 system is made up of two parts, the Gal4 gene which is the transcription activator gene, and the UAS which is the upstream activation sequence. UAS functions as a binding site for Gal4 and thus, the existence of UAS directs where exactly the Gal4 gene will be inserted into a genome. The specific gene that needs to be activated is called the reporter gene and is located right after UAS. Typical reporter genes can be genes encoding fluorescent proteins, or channelrhodopsin. It should be noted that the presence of the Gal4 gene alone in a genome has no or little effect on cells and organisms. The combination of Gal4 and UAS enables the expression of downstream genes and therefore, the reporter genes can be expressed and desired proteins can be produced.[187] To generate the Gal4-UAS system in *Drosophila*, UAS-containing virgin female flies are typically crossed with Gal4-carrying male flies, and the resulting offspring have the Gal4-UAS present in the genome.

One popular application of the Gal4-UAS system is in optogenetics, where light-sensitive ion channels such as channelrhodopsin are expressed to stimulate neurotransmitter release from neurons with high temporal and

spatial precision. Channelrhodopsin is sensitive to a specific wavelength of light. When the light is absent, it is inactive. With the presence of the specific light to activate a particular channelrhodopsin, it changes conformation to enable rapid flow of various cations to strongly depolarize neurons. Using channelrhodopsin as the reporter gene, it is possible to utilize the Gal4-UAS system to study chemical signaling among specific types of neurons, e.g. dopamine, serotonin, or octopamine neurons, in brain or NMJ of *Drosophila* larva.[109, 188-190] The channelrhodopsin allows stimulation of specific neurons without stimulating muscles as well as other cells spatially close to the neurons of interest.

3.4 Other Biological Models

In addition to the three biological model systems that have been used for this thesis work, many other biological models are also available and have led to breakthroughs for neuroscience research. The simplest models are *in vitro* and *ex vivo* preparations, which contain cell cultures, and preparations of organotypics and brain slices. Healthy neuroendocrine cells are one type of cell culture and common examples are chromaffin cells from adrenal glands, enterochromaffin cells from intestines, and beta cells from pancreatic islets. These cells are isolated from the endocrine system and secrete norepinephrine, serotonin, or similar substances upon receiving signal input from the nervous system. Another type of cell culture, which resembles healthy neuroendocrine cells, is that derived from cancer cell lines which are isolated from neuroendocrine tumors. These cells commonly originate from the adrenal glands (pheochromocymota, PC12 cells), intestines (carcinoid tumors, BON cells), and pancreas (insulinoma, INS-1 cells). Transmitters secreted by these cell lines are similar to the healthy neuroendocrine cells, except that PC12 cells release predominantly dopamine instead of norepinephrine. The third type of cell culture consists of neurons. The common neuronal cultures include cortical, hippocampal, and spinal neurons, as well as neuronal stem cells. Organotypics and brain slices are typically prepared using brain or spinal cord from mice or rats.

A variety of other invertebrates with simple nervous systems or large-sized neurons are considered useful model systems for neuroscience research apart from *Drosophila melanogaster*. These include the round worm *Caenorhabditis elegans* used for neuronal development and behavior studies, *Aplysia californica* used for plasticity-related research, the pond snail *Lymnaea stagnalis* used for studies of learning and memory as well as neuronal regeneration, and *Sepia officinalis*. In the case of vertebrates, several

species of fish, such as *Astatotilapia burtoni* and the zebrafish *Danio rerio*, are frequently used models in terms of understanding nervous system disorders and social behaviors. The clawed frog *Xenopus*, including two main model species *Xenopus laevis* and *Xenopus tropicalis*, is widely preferred in research related to signal transduction. The frog NMJ is a famous model system for understanding structure and function of synapses as well as investigating mechanism underlying synaptic activities. Mammals such as mice and rats are perhaps the most commonly used models in neuroscience field and a great number of *in vivo* measurements have been performed in these two models.

It is important to choose appropriate biological models for specific research questions. Knowing the advantages and limitations of each model system, the next is to use relevant techniques to investigate scientific questions. Chapter 4 will introduce several techniques that have been developed and widely applied to study exocytosis.

Chapter 4. Techniques to Monitor Exocytosis

Since exocytosis is a highly important biological process, developing suitable techniques to measure and visualize exocytosis is thus of great importance. A couple of methods within the areas of electrochemistry, electrophysiology, and imaging have made it possible to study specific aspects related to exocytosis. This includes quantification of neurotransmitter release from single vesicles, measurement of extracellular neurotransmitter concentration, monitoring vesicle fusion, as well as visualization of vesicle movements and activity of the fusion pore. The main focus of this chapter is to introduce in detail the techniques that have been used to study exocytosis during this thesis work. In addition, introduction regarding several other traditional and frequently employed techniques is also included.

4.1 Brief Background of Electrochemistry in or at Cells

In the 1980s, carbon microelectrodes with a tip diameter of 0.5-2 μm were fabricated by Meulemans et al. and employed to measure endogenous ascorbic acid concentration in neurons of *Aplysia californica* with intracellular voltammetry.[191] This is one of the earliest reports of electrochemical measurements from single cells. One year afterwards, the same technique was utilized to study the intracellular concentration of serotonin in metacerebral cells.[192] In addition, detection of intracellular dopamine concentration in neurons of *Planorbis corneus* was accomplished using carbon ring electrodes in 1988.[193] Before the birth of amperometry, most electrochemical studies focused on the use of voltammetry to follow concentration changes of interested intracellular chemical species.

In the early 1990s, amperometry was developed by the Wightman group to study individual exocytotic release events from single bovine chromaffin cells using disk-shaped microelectrodes.[194] By combining amperometry with cyclic voltammetry, it has been confirmed that the release events detected by amperometry are indeed oxidized catecholamine molecules which are secreted during exocytosis.[195] In 1992, the presence of ¨foot¨ in amperometric measurements was reported by Chow et al. and interpreted as the formation of a small-sized fusion pore at the beginning of some release events.[196] The successful combination of patch clamp and amperometry gave rise to the invention of the patch amperometry technique in 1997, enabling simultaneous detection of vesicle fusion and neurotransmitter release.[61] In 2015, the Ewing group developed a method called intracellular vesicle impact electrochemical cytometry (IVIEC) to directly quantify the amount of catecholamines stored inside single vesicles within a living cell.[107]

Quantification of the amount of reactive oxygen and nitrogen species (ROS/RNS) as well as intracellular production rates of four main ROS/RNS species in single phagolysosomes of macrophages was achieved in 2017 and 2019, respectively.[197, 198]

4.2 Single Cell Amperometry (SCA)

In response to external stimuli, secretory vesicles are capable of undergoing exocytosis to release various neurotransmitters and hormones. Since exocytosis occurs on the time scale of millisecond or submillisecond and the amount of release from individual vesicles is typically around the level of zepto- to femtomole, developing techniques with sufficient temporal resolution and sensitivity is critical. SCA is an electrochemical technique that enables direct quantification of exocytotic release. It offers high sensitivity and sub-millisecond temporal resolution, making it possible to resolve and follow individual release events. Therefore, since its introduction in 1990, SCA has been a powerful and popular technique in the research field of exocytosis.

To be detected by amperometry, neurotransmitters are required to be electroactive, which is the ability to be oxidized or reduced at a certain potential. A variety of transmitters are electroactive, including catecholamines, serotonin, and histamine. However, many other transmitters such as glutamate, acetylcholine, and neuropeptides are non-electroactive which limits the direct application of SCA to a greater extend. To solve this problem, biosensors with surfaces being modified by specific enzymes have been developed for the measurement of some non-electroactive molecules.[199] Another feasible way is to load secretory vesicles with one type of electroactive transmitter, which is typically co-released with the non-electroactive species during exocytosis. Amperometric detection of the introduced electroactive transmitters might offer some useful insights regarding the exocytosis of the non-electroactive molecules.[200] Selectivity is the other limitation of amperometry. In the case of catecholamine transmitters, dopamine, norepinephrine, and epinephrine have similar structures and so as their oxidation potentials. With amperometry, it is difficult to specifically measure one of them without the interference from the other two.

Figure 11A shows a diagram for use of SCA to measure exocytosis from a single cell. A carbon fiber microelectrode is typically used as a working electrode and placed on top of a single cell. Since the working electrode resembles the function of a receiving cell, this arrangement can be viewed as a simplified structure of synapse. A constant potential, enough to oxidize the

interested transmitter species, is applied to the working electrode relative to an Ag/AgCl reference electrode. Exocytosis is typically triggered chemically (with e.g. K^+, nicotine, Ba^{2+}, etc.), but can also be initiated electrically or mechanically. As depicted in Figure 11A, chemical stimulation can be introduced by placing a glass pipette loaded with stimulation solution next to the cell. A commonly used stimulation solution is the high K^+ stimulation solution. At the resting stage, the cell is bathed in isotonic solution which mimics the extracellular ionic environment. The isotonic solution consists of a low concentration of K^+ and a high concentration of Na^+. In contrast, the high K^+ stimulation solution is made up of a high concentration of K^+ and a low concentration of Na^+. The application of the stimulation solution can directly disrupt the ionic gradients across the cell membrane to induce membrane depolarization and therefore, trigger neurotransmitter release. Since the transmitter concentration at the electrode surface is 0, the released transmitters diffuse rapidly across the gap between the cell and the electrode. Upon reaching the electrode surface, the transmitters are immediately oxidized (redox reactions shown in Figure 11A) and this process results in current transients at the electrode.[194] The sampling rate for amperometric measurements can be adjusted. Sufficiently high sampling rate enables the detection of individual exocytotic release events. A typical amperometric current-time trace measured from a single cell contains a train of several current transients, which are also called amperometric spikes, and each spike corresponds to exocytosis from one vesicle.

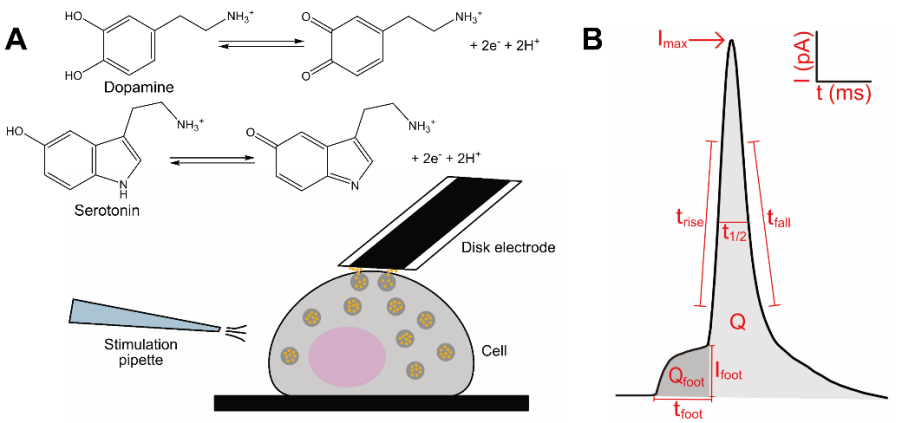

Figure 11. A. Scheme showing the basic SCA protocol for measuring exocytosis from a single cell. A disk microelectrode is placed on top of the cell, and a stimulation pipette is used to stimulate exocytosis. The redox reactions for dopamine and serotonin are included. B. Illustration of an amperometric spike with different parameters, including Q, I_{max}, $t_{1/2}$, t_{rise}, t_{fall}, I_{foot}, t_{foot}, and Q_{foot}.

4.2.1 Dynamics of Exocytosis Revealed by SCA

Quantitative and dynamic information regarding each exocytotic release event can be revealed by analyzing individual amperometric spikes. The Igor Pro software program is commonly used for processing amperometric data, and the instruction of data analysis has been published by Mosharov and Sulzer.[201] Briefly, background of an amperometric trace is first defined by selecting a 3-s trace section without any spikes. According to the noise level of the defined background, criteria for exocytotic spike as well as feet (discussed below) can be decided and used by the software to select spikes.

A representative amperometric spike is shown in Figure 11B. Several parameters of the spike can be examined and important information about exocytotic process can be obtained.[201, 202] Q is the charge of the spike and is calculated from the time integral of the spike. It can be subsequently used to calculate the number of molecules released from each exocytotic event with Faraday's law, offering quantitative aspect of exocytosis. In Faraday's law ($N = Q/nF$), in addition to Q, N is the mole amount of neurotransmitters oxidized on the electrode, n is the number of electrons transferred in the oxidation reaction (2 electrons for the oxidation of cateholamines and serotonin), and F is the Faraday constant (96485 $Cmol^{-1}$). I_{max} is the spike height measured from the baseline to the highest point of the spike. The exact meaning of I_{max} in exocytosis is not well characterized, but one possibility is an indication of the fusion pore size.

The other three parameters of the main spike, $t_{1/2}$, t_{rise}, and t_{fall}, are related to the time course of the release event. During exocytosis, a fusion pore is formed between the vesicle and the cell membrane. The continuous dilation of the fusion pore enables the release of neurotransmitters. Upon reaching the electrode surface, the released transmitters are rapidly oxidized which leads to a sudden increase of the measured current. This current increase is shown as the rising phase on the amperometric spike, followed by a slower falling phase.[104, 203-205] Therefore, $t_{1/2}$, which is the width of the amperometric spike at its half height, represents the duration or the stability of the fusion pore. The parameter t_{rise} is the time spent to rise from 25% to 75% of the height on the rising phase of the spike, and represents the duration of the fusion pore opening. The parameter t_{fall} is the time required to fall from 75% to 25% of the height on the falling phase of the spike, and indicates the duration of both transmitter diffusion and fusion pore closing.

Exocytotic spikes can be divided into two categories based on whether they possess a single- or double-exponential decay on the falling phase. A single-exponential decay indicates that the falling phase of the amperometric spike is predominantly controlled by one factor, which is the diffusion of released transmitters. A double-exponential decay, on the other hand, suggests the existence of another factor, in addition to diffusion, that affects the falling phase of the spike. This factor has been proposed to be the closing of the fusion pore. By inhibiting the protein actin, the decreased fraction of spikes with a double-exponential decay suggests the possible involvement of actin in the closing of the fusion pore.[124] In contrast, the inhibition of dynamin results in a higher percentage of spikes with a double-exponential decay, indicating the role of dynamin in stabilizing the fusion pore.[123]

Traditionally, carbon fiber disk microelectrodes have been used to perform SCA.[194] The surface areas among different batches of disk electrodes remain relatively the same, making the comparison of amperometric data easy. However, other types of electrodes have also been applied to measure exocytosis in some circumstances. The exocytotic release detected by flame-etched nanotip microelectrodes was compared to the result obtained by disk electrodes (**paper III**). Although showing slightly different release dynamics, the quantification is unchanged between these two types of electrodes. The observed difference in release dynamics has been suggested to arise mainly from the altered distance between the electrode and the cell when using nanotip instead of disk electrodes.[206] In addition, exocytosis from different positions on the surface of a cell has been monitored with SCA. Release from top or bottom of a cell has been shown to have distinct frequency and release dynamics.[207] The exocytotic hot spots, positions on a cell membrane where exocytosis frequently occurs, have been electrochemically mapped with microelectrode arrays.[208, 209]

4.2.2 Pre- and Post-Spike Feet

A relatively small current increase at the beginning of the rising phase on the amperometric spike can be seen in Figure 11B, which is referred to as the ‘pre-spike foot’. The foot is normally present in some of the amperometric spikes with a shape of a plateau or a slope. The concept of the foot was first proposed in a paper published in 1992 by Neher and coworkers, in which they applied both amperometry and patch-clamp to examine vesicle fusion in chromaffin cells.[196] Among all exocytotic release events from chromaffin cells, the percentage of spikes with a foot was estimated to be 70% by Zhou et al.[210] Additionally, a special type of foot called a ‘stand-alone foot’ has been reported, meaning that the foot alone can be an entire release event and the

subsequent dilation of the fusion pore is not necessarily required to accomplish exocytosis. The existence of a 'stand-alone foot' was supported by another study using the patch amperometry technique.[61] Although not every release event is accompanied by a pre-spike foot, characteristics of the feet might offer useful information in terms of vesicle properties, making this an important factor when it comes to studying exocytosis.[204]

The analysis of pre-spike foot closely resembles how the exocytotic main spike is analyzed.[201] Three parameters including I_{foot}, t_{foot}, and Q_{foot} are used to provide quantitative and dynamic information about neurotransmitter release via the foot (Figure 11B). The foot has been interpreted as an initially formed fusion pore with a relatively small pore size in comparison to the fusion pore during the main release event. Through this small pore, some neurotransmitters escape to the extracellular space. I_{foot} is the maximum current of the foot and might be related to the size of the initial pore. A plateau-shaped foot suggests that the pore size is nearly constant, whereas a slope-shaped foot shows an increase or decrease of the pore size. The parameter t_{foot} is the width of the foot and represents the duration or stability of the initial pore. The parameter Q_{foot} is the charge of the foot and using Faraday's law, it can be converted to the number of transmitter molecules released during the foot.

In addition to the pre-spike foot, another foot called a 'post-spike foot' has been reported by Mellander et al. in 2012.[211] The pre-spike foot is found before the main exocytotic event, whereas the post-spike foot is occasionally present at the end of the falling phase on the amperometric spike. The observation of post-spike feet suggests the possibility for the exocytotic fusion pore to be constricted to a relatively small size and subsequently closed to terminate exocytosis. The current of the post-spike foot is lower than the pre-pike foot as some transmitters have been released during the main exocytotic event. Therefore, the transmitter concentration inside the vesicle is lower after the main release event when compared to before. The coexistence of pre- and post-spike feet has been discovered in some amperometric spikes, indicating that exocytosis involves the formation of an initial small fusion pore, which first expands and then constricts to a small size again, and eventually closes.

4.2.3 Modeling Exocytosis

Amperometry is a highly useful technique when it comes to quantifying exocytosis. However, amperometric data contain a large amount of information and can be sometimes difficult to interpret. Therefore, modeling methods have been applied to amperometric data in order to achieve

a better understanding regarding exocytosis. Several modeling methods and their applications are briefly introduced here.

The finite element method is a numerical modeling approach typically used to solve problems related to structural analysis, mass transport, heat transfer, etc. It has been applied to compare between dopamine diffusion that occurs closely to the cell membrane and diffusion in a simple buffer.[212] The glycocalyx surrounding the cell has been suggested to slow down the diffusion process. The same method was utilized to study potential factors causing differences between amperometric spikes measured by nanotip and disk electrodes (**paper III**). Additionally, the finite element method has also been used to simulate serotonin release from platelet granules and the presence of a dense-core structure inside the granules was demonstrated.[213] Another modeling method is the random walk simulation, which is relatively simple. It was developed to understand the Brownian motion, and can be applied to predict outcomes of complex systems based on diffusion of particles. This method was utilized to simulate neurotransmitter release from vesicles, where diffusion is spatially restricted, to understand the three different stages of exocytotic events revealed by amperometric data.[214] It is also useful in terms of localizing exocytotic release sites on the cell surface based on different spike shapes.[209] A third modeling method is theoretical analysis, which has been applied to study the maximum aperture angle that a vesicle needs to undergo full release, the size and the energy of the initial fusion pore, the influence of dense-core structure on vesicular transmitter storage and distribution, and the possible relationship between vesicle inner structure and exocytotic release dynamics.[66, 104, 205, 215]

4.2.4 A Few Representative Applications of Amperometry in Neuroscience Research

Owing to the ideal temporal resolution and high sensitivity, as well as the ability to quantify exocytosis, amperometry is widely applicable for studies aiming to understand the basics of exocytosis. It has been used to quantify catecholamine release from LDCVs in PC12 cells and from SSVs in cultured neurons.[148, 216, 217] The release of norepinephrine from single synapses has also been successfully probed and measured with amperometry.[218] In addition to exocytosis of catecholamines, serotonin release from cultured leech neurons and octopamine release from NMJ varicosities in *Drosophila* larvae have been investigated and reported.[190, 219] The co-release of catecholamines and ATP molecules from same vesicles in chromaffin cells has been studied by the combination of amperometry and electrophysiological measurements.[220] It should be noted that the applications mentioned above

only represent a few examples and there are a great number of other studies utilizing amperometry as one of the research methods.

4.3 Vesicle Impact Electrochemical Cytometry (VIEC) and Intracellular Vesicle Impact Electrochemical Cytometry (IVIEC)

In 2009, Omiatek and coworkers developed a technique termed flow-VIEC which utilizes a microfluidic device to detect single dopamine-containing liposomes.[221] Inside the device, liposomes are first separated by capillary electrophoresis and then detected. A carbon microelectrode is placed at the end of the device, functioning to oxidize and quantify the number of dopamine molecules inside individual liposomes which are lysed prior to reaching the electrode. The device was applied one year later to probe catecholamine storage in single vesicles isolated from PC12 cells.[117] Interestingly, they found that the catecholamine storage inside PC12 vesicles measured by the device was much larger than the vesicular release quantified by SCA, indicating the possibility for vesicles not to release all their transmitter content during exocytosis, the partial release scenario. However, fabrication of the microfluidic device can be difficult and time-consuming, thus limiting the application of flow-VIEC.

A more practical VIEC technique was developed in 2015 by Dunevall et al. to quantify transmitter content in single vesicles.[222] Upon isolating vesicles from cells or tissues, detection was achieved by placing a 33 μm carbon fiber disk microelectrode directly into a suspension of vesicles, allowing the vesicles to adsorb onto the electrode surface (Figure 12A). Due to the potential applied to the electrode, the adsorbed vesicles rupture and their transmitters are directly detected by the electrode, which are shown as amperometric spikes. Each spike corresponds to rupturing of one vesicle. Using Faraday's law, the number of transmitter molecules stored in individual vesicles can be calculated from the charge of the spike (same as analyzing SCA data). More importantly, approximately 86% of all amperometric events measured by VIEC are shown to come from rupturing of single vesicles, instead of several vesicles rupturing and being quantified at the same time. This validates the use of the VIEC technique for measuring single vesicles. The development of VIEC allows us to count transmitter molecules inside vesicles and compare it to how many are released during exocytosis.

One advantage of VIEC is the ability to investigate the effects of drug treatments specifically on vesicle properties, e.g. vesicle membrane and vesicle proteins, without interference from other cellular components. However, when it comes to understanding the natural behaviors of vesicles

within their original environment, interactions between vesicles and other organelles as well as the cell membrane are highly important factors. Additionally, the vesicle isolation process involves many steps which might cause some alterations in the vesicle properties. Leakage of vesicular transmitters during the isolation process is one problem, which leads to some uncertainties when making direct comparison between VIEC data and exocytotic data quantified by SCA. Therefore, it is of great need to develop a technique which enables direct quantification of vesicular transmitters within the cellular environment.

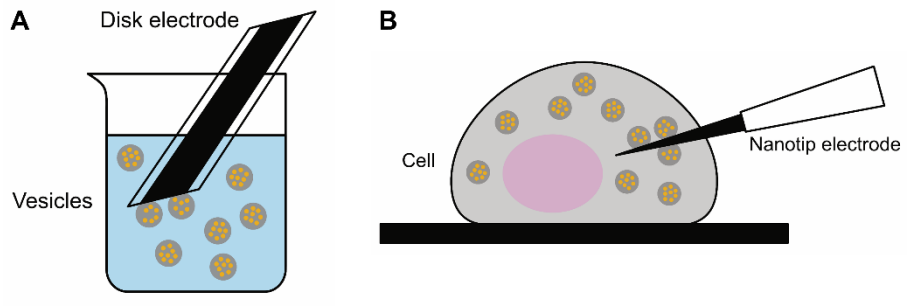

Figure 12. Schemes showing A. measurement by VIEC in a solution of isolated vesicles with a 33 µm disk microelectrode, and B. measurement of IVIEC in a single cell with a nanotip electrode.

In 2015, an intracellular VIEC (IVIEC) technique was developed by Li et al., which fulfills the idea to directly probe vesicles within their natural environment.[107] By flame-etching a carbon fiber microelectrode, a nanometer-scale tip was obtained and utilized to insert into the cell. The sharp tip of the electrode minimizes the damage caused to the cell membrane during the insertion process, which ensures the viability of the cell during the entire measurement. Once the electrode tip reaches the cytoplasm, vesicles inside the cytoplasm adsorb onto the surface of the tip and rupture to release their transmitter molecules, which are measured as amperometric spikes. Each spike represents the oxidation of transmitter molecules from one vesicle and the charge of the spike can be used to calculate the amount of transmitters being stored in that vesicle. Figure 12B illustrates how IVIEC is carried out with a nanotip electrode. By comparing the exocytotic result to the IVIEC result measured with the same electrode, it was shown that during a regular exocytosis, a vesicle releases around 64% of its entire transmitter storage, supporting the theory of partial release.

4.3.1 Proposed Mechanisms for VIEC and IVIEC

The advent of VIEC and IVIEC techniques makes quantification of vesicular transmitter storage possible. However, mechanisms underlying these two techniques remain to be elucidated. These includes how vesicles rupture and open to allow the transmitters inside to be quantified by the electrodes, and what factors might regulate vesicle opening during VIEC and IVIEC measurements. Lovrić et al. proposed that after a vesicle is adsorbed onto the electrode surface, the potential applied to the electrode is sufficient to electroporate the vesicle membrane, thus opening a pore between the vesicle and the electrode.[223] Transmitters inside the vesicle can diffuse out through this pore and are then detected at the electrode. Higher potential or temperature leads to increased possibility of vesicle rupturing and opening.[224] In addition, it was observed that vesicles with larger size tend to rupture earlier and much easier in comparison to the ones with smaller size.

However, the existence of large amount of vesicular membrane proteins hinders vesicles from getting close enough to the electrode, which increases the difficulty for electroporation. By comparing to liposome models with no or little amount of membrane proteins, it was suggested that the migration of vesicular membrane proteins away from the electrode contact region allows the vesicle to get close to the electrode, which is essential for facilitating electroporation during VIEC and IVIEC.[223, 224] Excited fluorophores and dimethyl sulfoxide (DMSO) are capable of increasing the fragility and permeability of the membrane by inducing oxidation of membrane lipids. By conjugating excited fluorophores to vesicular membrane lipids or simply by incubating vesicles with DMSO, the frequency of vesicle rupturing and opening was observed to be increased, again demonstrating electroporation as the possible mechanism underlying VIEC and IVIEC measurements.[225]

Another two important questions regarding VIEC and IVIEC are the location of the pore on the vesicle and collection efficiency, i.e. what fraction of transmitters from a vesicle actually reach the electrode surface and are measured, which determines the precision of these two techniques. Li et al. applied quantitative modeling to examine these two questions and found that for VIEC measurements with 33 μm carbon fiber disk microelectrode, all transmitter content inside the vesicle can be quantified regardless of where the pore opens on the vesicle.[226] For IVIEC measurements, the collection efficiency is highly critical as the electrode tip that is inserted into the cytoplasm to quantify transmitter content is much smaller than the disk electrode used for VIEC. Moreover, since the electrode tip is typically a

conical shape, the back of the tip is relatively larger than the front, and the location of adsorption along the tapered electrode might also affect the collection efficiency. To understand this, the diffusion of transmitter molecules out of a vesicle was simulated under various positions along the electrode from shank to tip. Simulations show that near the electrode tip only a small portion of the transmitter from a vesicle is oxidized if the vesicle pore opens opposite the electrode. Since the amount quantified by IVIEC is the same when compared to disk electrode in VIEC, this suggests the position of the pore in IVIEC to be at the electrode surface and thus, the ability of IVIEC to directly assess vesicular transmitter content is validated.

4.3.2 Applications of VIEC and IVIEC

The two electrochemical techniques, VIEC and IVIEC, have improved our understanding regarding the fundamentals of cellular communication. The combination of SCA and IVIEC to study transmitter release and storage supports partial release as one mode of exocytosis, during which vesicles release only a fraction of the transmitter load via an open-and-closed fusion pore. The fraction of release, calculated by the amount of released transmitter over that stored, can be altered by a variety of drug treatments as well as cellular activity.

Using SCA and IVIEC, it has been reported that zinc or MPH incubation leads to an increase of the release fraction (**paper I**).[227] The same trend was observed after a cell is being repetitively stimulated, a way to induce activity-dependent plasticity (**paper II**). Conversely, cells incubated with cocaine have a decreased release fraction (**paper I**). Since zinc and MPH have a positive effect on learning and memory, whereas cocaine is the opposite, these studies suggest that fraction of release might be an indicator in learning and memory as well as involved in the formation of plasticity. In addition, some anticancer drugs and anesthetics have also been investigated with combined SCA and IVIEC. Interestingly, cisplatin, tamoxifen, and lidocaine alter neurotransmission via different mechanisms at low versus high concentrations.[119-121] The effect of extracellular ATP on exocytosis was examined using SCA, IVIEC, and VIEC, and the role of purinergic receptors in shifting the mode of exocytosis from partial to full release was indicated.[228] The use of VIEC in this study makes it possible to pinpoint observations particularly to the vesicular level without the interference of cellular machinery. Another application regarding IVIEC is the simultaneous detection of exocytotic release and vesicular transmitter storage (**paper VI**). This was achieved by employing two electrodes to measure SCA and IVIEC

from a single cell, providing some interesting insights about the dynamic alteration of vesicular storage induced by stimulation.

In addition to studying the mechanism of exocytosis, IVIEC, or a scenario similar to IVIEC, has also been applied to monitor ROS/RNS levels in single cells. By inserting a cylindrical nanowire electrode into a living macrophage, the entire amount of ROS/RNS in single phagolysosomes is oxidized on the platinum-coated nanowire and directly quantified.[197] The real-time production rates of four main species of ROS/RNS inside individual phagolysosomes have been determined using platinized carbon nanoelectrodes.[198]

4.4 Patch Clamp

The patch clamp technique is an electrophysiological technique pioneered by Neher and Sakmann in the late 1970s. It was developed to monitor ion fluxes through single ion channels in order to understand their functions in the processes of action potentials and other neuronal activities.[229] It was later adapted to record membrane capacitance changes caused by vesicle fusion and retrieval during exocytosis and endocytosis, respectively, in chromaffin cells.[230] Since then, patch clamp has become one of the traditional techniques frequently used to monitor exocytosis as well as endocytosis.

The basic components of patch clamp consist of a hollow glass micropipette, or also called patch pipette, which contains an electrolyte solution inside, a recording electrode which is placed inside the micropipette and connected to an amplifier, and a reference electrode which is positioned in the bathing solution around the cell to be measured. Several modes of patch clamp have been developed to meet different research purposes. Among them, cell-attached patch and whole-cell patch are the two most commonly used ones. In the cell-attached patch, the micropipette is brought closely to the cell membrane to form a gigaseal, a seal with a high resistance (on the order of a gigaohm), without causing any damage to the membrane. The current flow due to the activities of one or several ion channels in the patched membrane can thus be recorded by the electrode inside the pipette. Since the cell membrane remains intact under this mode, intracellular structures and cellular activities which normally alter ion channel activities are not affected.[231, 232] Therefore, real-time measurement of ion channel activities can be achieved.

In the whole-cell patch, a strong suction force or a large current pulse is applied to the micropipette to break the cell membrane. Thus, the electrolyte

solution within the pipette has direct access to the intracellular environment, and real-time cellular changes induced by drug treatments can be studied.[233] In contrast to the cell-attached patch, the recording electrode in the whole-cell patch forms a circuit with nearly the entire membrane, and currents through many ion channels can be simultaneously monitored. Since the volume of the pipette solution is typically larger than the cell volume, soluble cell contents are slowly replaced by the pipette solution. Therefore, limited recording time is a drawback of the whole-cell patch.

Patch clamp can be performed using either voltage-clamp or current-clamp. In voltage-clamp, the voltage across the cell membrane is controlled while current changes are monitored. In current-clamp, on the other hand, the current across the membrane is controlled and voltage changes are recorded. The measurement of membrane capacitance is done by use of voltage-clamp.[230] Changes of membrane surface area are shown as capacitance fluctuations and correlated with membrane gain or loss from vesicle fusion during exocytosis or vesicle retrieval during endocytosis, respectively. The capacitance changes measured can be used to estimate the size of each fused vesicle. Therefore, patch clamp can even be used to study aspects of exocytosis of non-electroactive neurotransmitters. The ability to monitor both exocytosis and endocytosis makes it possible to follow a longer period in the life cycle of a vesicle.[234] However, patch clamp cannot offer quantitative information about exocytosis, nor can it be used to measure specific transmitters. Moreover, the signal-to-noise ratio of patch clamp recordings might hinder the detection of small vesicles and vesicles undergoing partial release. The suction force applied to the cell membrane might also affect the stability of the fusion pore formed within the area of the patched membrane, resulting in the observation of full release more frequently.

4.5 Patch Amperometry

The inability of patch clamp to quantify exocytotic release has been solved by another technique called patch amperometry, which was developed by Albillos et al. in 1997.[61] By inserting a carbon fiber microelectrode inside the micropipette used for patch clamp measurement, individual vesicle fusion events and the resulting neurotransmitter release can be simultaneously monitored. The patch amperometry system is similar to that for patch clamp, and the measurements are typically performed under the cell-attached mode. Upon forming a gigaseal between the pipette and a small region of the cell membrane, vesicle fusion events which occur within the region of the patched membrane are recorded as capacitance steps. The fused vesicles secrete transmitter molecules into the pipette. The electrode positioned inside the

pipette can thus be used to quantify exocytotic release from these fused vesicles, which are recorded as amperometric spikes. The direct correlation between capacitance steps and amperometric spikes confirms that every vesicle fusion event leads to the release of transmitter molecules.[235] The estimation of vesicle size from capacitance measurements and quantification of exocytotic release from amperometric results lead to a conclusion that vesicles contain roughly the same concentration of transmitters, regardless of the size. This of course assumes most release events measured here are full release. In some cases, a small increase followed by a larger increase of the capacitance was observed, and the correlated amperometric spike showed a 'foot' signal preceding the main spike. The foot is interpreted as a transient fusion pore with diameter less than 3 nm.

Patch amperometry was further modified to detect catecholamine concentration inside the cytosol, leading to the development of a technique named intracellular patch electrochemistry (IPE).[236] IPE is performed in the whole-cell patch mode, which allows the electrode inside the micropipette to have direct access to the cytosolic molecules, including catecholamines. IPE has two detection modes, an amperometric mode and a cyclic voltammetric (CV) mode. The amperometric mode measures the total amount of oxidizable molecules inside the cytosol, which includes catecholamines, their precursors (e.g. L-DOPA), their metabolites (e.g. dihydroxyphenylethylene glycol, 3,4-dihydroxyphenylacetic acid), and ascorbic acid. The CV mode, on the other hand, can be applied to identify catecholamines among all the molecules measured in the amperometric mode since they have special oxidation-reduction voltammograms. The results showed that cytosol has around 50-500 μM catechols (catecholamines, precursors, and metabolites), within which catecholamines only take up approximately 10%. The estimated cytosolic catecholamine concentration done by IPE is consistent with a previous study in *Planorbis corneus*.[237]

4.6 Fast Scan Cyclic Voltammetry (FSCV)

In CV, the potential applied on an electrode is scanned between two different potentials and the resulting current is measured. This current is then plotted versus the potential to obtain a voltammogram. The peak position inside the voltammogram indicates where reduction or oxidation of a certain compound happens. Since many electroactive molecules have their special redox potentials, CV is commonly used to identify and characterize specific molecules, the qualitative property of CV. The quantitative property can be achieved by examining the current at the redox peaks, which correlates with the concentration of the molecule being detected. A typical scan rate for CV

is 100 mV/s. However, to measure biological processes such as exocytosis, which occur in milliseconds, increased temporal resolution is required.

The development of FSCV brings the scan rate up to 400-1000 V/s which can be used to follow the dynamic alteration of neurotransmitter concentration *in vivo*.[238, 239] Although increased scan rate offers sufficient temporal resolution, a large background charging current is meanwhile generated due to the rearrangement of the electrode-solution interface when a potential is rapidly changed. The presence of the charging current can overwhelm signals measured from the molecule of interest and thus, background subtraction is needed.[240] The resulting redox currents are normally plotted against time in FSCV and the concentration change of the interested molecule in relation to time can be observed.

In terms of the element of selectivity, an advantage of FSCV over amperometry regarding studying neurotransmission is the ability to identify the transmitter of interest. A variety of special waveforms have been developed to target specific types of transmitters, including serotonin, dopamine, histamine, and octopamine.[241-244] Waveforms for measuring other intracellular molecules such as adenosine and guanosine are also available.[245] More importantly, FSCV has been employed to distinguish among the three types of catecholamine transmitters, which possess similar structures and redox potentials.[246, 247] The main biological application of FSCV is currently for *in vivo* measurements. Although the temporal resolution is not as great as amperometry, FSCV is still capable of catching subtle and rapid changes when performing measurements inside the brain. The good selectivity offered by FSCV is another essential point regarding *in vivo* measurements due to the complicated measurement environment and the presence of many interferences.

4.7 Fluorescence Imaging

Although the electrochemical and electrophysiological techniques described above are highly useful for the quantification of exocytosis, spatial imaging with these techniques is difficult when compared to imaging approaches. As introduced in section 2.2, a large number of proteins are involved in the process of exocytosis and dysfunction of these proteins can result in severe interruption of neurotransmission. In addition to proteins, Ca^{2+} is another important regulator in exocytosis as an increase of the cytosolic Ca^{2+} level is required for fusion between vesicles and the cell membrane. Therefore, visualizing the spatial distribution and transportation of certain proteins, as

well as following the real-time intracellular changes of Ca^{2+}, with imaging techniques adds a wealth of information to the understanding of exocytosis.

One of the most frequently used imaging techniques is fluorescence imaging. It is a non-invasive technique and requires labeling of the interested proteins or molecules with fluorescent probes (called fluorophores). The basic principle of fluorescence is the electronic transition of a molecule from a ground state to a higher excited state followed by the subsequent return to the ground state, during which a photon is emitted and measured as a fluorescent signal under a specific wavelength. The emission wavelength needs to be known prior to fluorescence imaging. It is typically longer than the excitation wavelength due to the energy loss caused by vibration and heat dissipation at the excited state.[248]

In many cases, fluorescent probes are small organic compounds or large natural proteins. Fluorescent false neurotransmitters (FFNs) are one type of organic fluorophore. Due to their high similarity to neurotransmitters such as monoamines, FFNs can be taken up by secretory vesicles and utilized to study transmitter release as well as uptake.[249] A typical example of a fluorescent protein is the green fluorescent protein (GFP), initially isolated from the jellyfish *Aequorea victoria*.[250] Since the discovery of GFP, various derivatives have been genetically engineered to optimize the properties of GFP as a fluorophore, e.g. fluorescence intensity, stability, etc.[251] One example of using GFP to study exocytosis is by binding GFP to another protein which is present in or on secretory vesicles, especially for LDCVs. Representative target proteins are chromogranins, the dense-core protein family, and neuropeptide Y which is stored in some LDCVs. This enables the real-time monitoring of vesicle activity and the resulting neuropeptide release.[252]

To measure the dynamic changes of fluorescent signals, fluorescence microscopes are typically employed. The relatively simple but most commonly used setup is the epifluorescence microscope, where both excitation and detection occur through the same light path. Using a dichroic mirror, the excitation light can be transmitted to the sample and meanwhile, only the emitted light as well as a small fraction of reflected excitation light is allowed to reach the detector, resulting in a high signal-to-noise ratio.[248] Another two more advanced setups are confocal microscopy and total internal reflection fluorescence (TIRF) microscopy. The key point of confocal microscopy is the use of a pinhole, which allows only the focused emitted light to reach the detector, to achieve a better contrast of the image.[248] In addition, the depth of focus in confocal microscopy is normally well-controlled and

only a small section of the sample is in focus at any time. TIRF microscopy utilizes an evanescent wave to excite fluorophores presented within a thin area of a sample.[248] As light has perpendicular electric and magnetic fields, during total reflection at angles beyond the critical angle, a component of the electromagnetic field penetrates approximately 100-200 nm past the reflection plane and can result in absorption and subsequent fluorescence. The short distance is due to the rapid decay of this evanescent wave and allows observation of cellular activities which occur close to the membrane of a cell (e.g. vesicle fusion during exocytosis) on the reflection surface. This gives rise to an increased signal-to-noise ratio, but meanwhile limits the application of TIRF microscopy. Additionally, it is worth mentioning that several super-resolution microscopy techniques, such as stimulated emission depletion (STED) microscopy, have been developed during the past several decades. By bypassing the diffraction limit which constrains the resolution of traditional light microscopy, super-resolution imaging can be achieved. In the field of exocytosis, STED microscopy has been applied to visualize the structure of SNARE complex and follow the dynamic behaviors of exocytotic fusion pore, both inside living cells.[106, 253]

4.7.1 Ca^{2+} Imaging to Study Exocytosis

One fluorescence imaging technique used for this thesis work has been Ca^{2+} imaging (**paper II**). Due to the vital role of Ca^{2+} in regulating exocytosis, many fluorescent probes have been developed to follow the dynamic change of intracellular Ca^{2+} level.[254] Due to the high photon yield, Fura-2 is one of the fluorophores frequently used for Ca^{2+} imaging.[255] Fura-2 is typically introduced into the cell via incubation or alternatively, by microinjection. In the case of incubation, an acetoxymethyl ester is linked to Fura-2 (Fura-2AM) to make it more hydrophobic, which promotes passive loading across the cell membrane. Moreover, DMSO can be co-incubated with Fura-2AM to increase the permeability of the cell membrane. When Fura-2AM reaches the cytoplasm, the acetoxymethyl ester is removed by intracellular esterases so that the remaining hydrophilic fluorophore cannot leave the cell. Without binding to Ca^{2+}, Fura-2 can be excited at a wavelength of 380 nm. The binding between Ca^{2+} and Fura-2 shifts the excitation wavelength to 340 nm, which allows ratiometric imaging.[256] The emission wavelength of Fura-2 is 510 nm regardless of Ca^{2+} binding and the emission ratio from the two excitation wavelength (340/380) is proportional to the intracellular Ca^{2+} level. Ratiometric imaging removes uncertainties caused by varied concentrations of fluorophores present among different cells. Therefore, it is particularly useful when comparing fluorescent measurements within a cell population.

The possibility to quantify and visualize exocytosis has greatly improved our understanding in cellular communication. The techniques described in this chapter have been employed to study various important aspects of exocytosis, such as neurotransmitter release and uptake, vesicle distribution and movement, exocytotic protein machinery, etc. In addition to these, membrane lipids also play essential role in exocytosis, especially during vesicle fusion. The next chapter will introduce several membrane lipids and their possible involvements in exocytosis. Imaging techniques used for studying membrane lipids will also be discussed.

Chapter 5. Membrane Lipids, Their Roles in Exocytosis, and Mass Spectrometry Imaging to Study Lipids

During exocytosis, the formation of a fusion pore between secretory vesicles and cell membrane is necessary for the release of neurotransmitters. This fusion process has long been considered to be mainly mediated by various proteins. However, the significant roles of membrane lipids in fusion as well as regulating exocytosis have been suggested recently. The main focus of this chapter is to introduce several membrane lipids, including phospholipids, fatty acids, and cholesterol, and how they are involved in regulating exocytosis. Mass spectrometry imaging, a group of imaging techniques frequently used to study membrane lipids, is also described in this chapter.

5.1 Cell Membrane Structure and Their Involvement in Exocytosis

The cell membrane, which is also called plasma membrane, serves as a biological barrier to separate the interior of a cell from the extracellular environment. This ensures cellular components and cellular activities to be unaffected, at least not in a direct manner, by the changes of the extracellular environment. It also serves as an interface for communication between the cell and its environment. The cell membrane consists of a lipid bilayer, on which a large number of membrane proteins are present, as illustrated in Figure 13. The lipid bilayer contains of three categories of amphipathic lipids which are phospholipids, glycolipids, and sterols. Lipid molecular structure typically consists of a hydrophilic head group and a hydrophobic tail. Thus, the arrangement of lipid molecules within the lipid bilayer makes the cell membrane impermeable to water-soluble molecules, and the transportation of substances across the cell membrane is controlled actively or passively. Proteins on the cell membrane are either anchored to the outer leaflet of the membrane (e.g. enzyme proteins) or integrated into the lipid bilayer (e.g. transporter proteins, ion channels). In addition, the inner leaflet of the cell membrane is attached to a network of protein fibers termed the cytoskeleton which functions to maintain the shape of the cell. The outer leaflet of the cell membrane, on the other hand, can be attached to the extracellular matrix and is therefore, important in the process of cell adhesion.

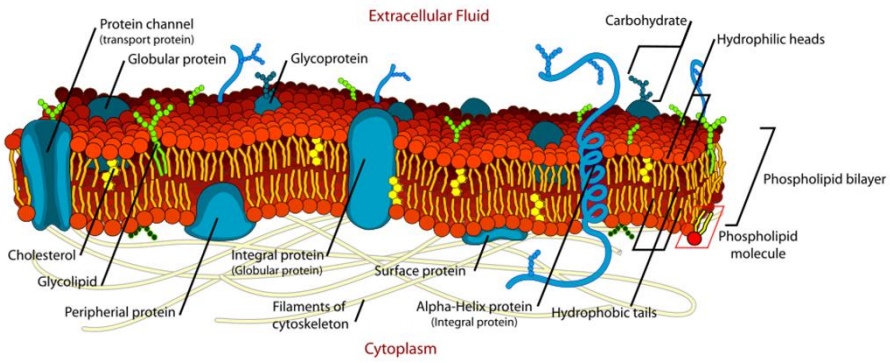

Figure 13. A diagram of the cell membrane showing a variety of lipids and proteins. Reproduced from https://commons.wikimedia.org/wiki/File:Cell_membrane_detailed_diagram_en.svg.

5.1.1 Phospholipids

Phospholipids are a major component of eukaryotic cell membranes. They contain a hydrophilic head with a negatively charged phosphate group, two hydrophobic tails made up of long-chain fatty acids and their derivatives, and a glycerol molecule which links the head group and the tails. Based on the substituent of the phosphate group, phospholipids can be classified into several subclasses, such as choline, ethanolamine, serine, inositol, etc. In addition to the different head groups, the number of carbon atoms and the degree of saturation on the fatty acid tails also determine the properties and functions of the phospholipids. Synthesis of phospholipids predominantly takes place at the membrane of the ER on the cytosolic side, thus facilitating the transport of phospholipids towards their destination in the cell.[257, 258] The newly synthesized phospholipids bud off from the ER membrane as a form of membrane vesicles, which are transported to and subsequently fused into the destined membranes. This enables transportation of membrane phospholipids within as well as between cells.

PC is one type of phospholipids which uses choline as the head group. It is the most abundant lipid species in eukaryotic cells, making up of approximately 45-55% of the total amount of lipids.[259] The head groups of PE and phosphatidylinositol (PI) are ethanolamine and inositol, respectively. Among all lipids in the cell, PE composes around 25% and is the second most abundant phospholipid species, whereas PI makes up about 10-15%.[259] Due to the presence of a positive charge on the choline or ethanolamine group and a negative charge on the phosphate group, PC and PE are zwitterionic lipids. PI, on the other hand, is negatively charged because of the phosphate group. The

biosynthesis of PC and PE in the ER mainly occurs via the condensation between diacylglycerol and cytidine diphosphocholine or cytidine phosphate-ethanolamine. The reaction is catalyzed by the enzyme diacylglycerol cholinephosphotransferase in the case of PC, or ethanolamine phosphotransferase in the case of PE.[260] Alternatively, PC can be synthesized via methylation of PE.[261] Another way to synthesize PE is through the phosphatidylserine decarboxylation pathway, which is the main synthetic pathway for producing PE in the membrane of mitochondria.[262] The biosynthesis of PI in the ER is achieved by the reaction between cytidine phosphor-diacyglycerol and myo-inositol, catalyzed by PI synthase.

To initiate exocytosis, a fusion pore needs to be generated between the vesicle and the cell membrane. The fusion pore is a highly curved structure and thus, phospholipids at the fusion sites need to be rearranged towards an increased curvature to promote the fusion process.[128, 129] Afterwards, the fusion pore expands to allow more transmitters to be released. The expansion of the fusion pore has been shown to be achieved by incorporating lipids.[127] The distribution of various lipid species within the phospholipid bilayer of the cell membrane is typically asymmetrical. PC is more concentrated in the outer leaflet of the cell membrane compared to the inner leaflet. Conversely, PE is preferentially located into the inner leaflet. Due to the relative geometrical sizes of the head group and the tails, PC, PE, and PI contribute differently to the curvature of the cell membrane. As shown in Figure 14, the head group and the two tails of PC take up similar spaces, making PC cylindrical-shaped. Therefore, PC can be found mostly in the flat or low curvature regions of the cell membrane. The head group of PE is relatively smaller compared to the two tails, making PE conical-shaped. PI, which has an inverted conical shape, has a larger head group than the two tails. Both PE and PI are considered to be present mainly in the high curvature regions of the cell membrane. The difference is that PE contributes to a negative curvature whereas PI contributes to a positive curvature. By introducing various exogenous phospholipids into the cell membrane, either via incubation or microinjection, the amount of transmitter release and the stability of the fusion pore during exocytosis are both affected.[131, 132] Additionally, altered levels of PC, PE, and PI in the cell membrane have been correlated with the stabilization of the fusion pore during repetitive stimulation (**paper V**), demonstrating the importance of phospholipids in regulating exocytosis.

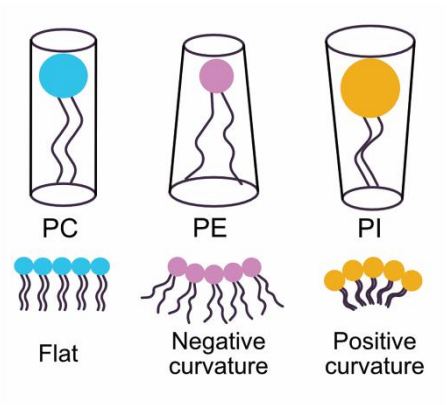

Figure 14. Structures and geometrical shapes of PC, PE, and PI. PC has a cylindrical shape and fits better in flat regions of the membrane. PE and PI have conical shapes and fit better in high curvature regions of the membrane.

5.1.2 Fatty Acids

The structure of fatty acids is made up of a carboxylic acid connected to a hydrocarbon chain, which can be saturated or unsaturated. The number of carbon atoms in the hydrocarbon chain is typically even and ranges from 4 to 28. Saturated fatty acids consist of no double bonds, while unsaturated fatty acids possess one or more double bonds, which are referred to as monounsaturated or polyunsaturated fatty acids, respectively. The double bonds in unsaturated fatty acids can be in either *cis* or *trans* configuration, which determines the flexibility of the carbon chain. The existence of a *cis* double bond bends the carbon chain, while a *trans* double bond does not give rise to such effect. With more double bonds being in the *cis* configuration, the carbon chain becomes highly bent and the conformational freedom of the fatty acid is limited. In the cell membrane, most fatty acid tails in the phospholipids have double bonds in the *cis* configuration. In comparison to the saturated or *trans*-unsaturated fatty acid tails, the bending effect induced by the *cis* double bonds makes it more difficult to tightly pack the *cis*-unsaturated fatty acid tails, resulting in an increased membrane fluidity.

The initial step of synthesizing fatty acids is the carboxylation of acetyl-coenzyme A (CoA) to malonyl-CoA with the presence of acetyl CoA carboxylase. The malonyl-CoA is then converted to ketoacyl-CoA via a condensation reaction with an acetyl-CoA. The ketoacyl-CoA is subsequently reduced, dehydrated, and hydrogenated to produce a new acyl-CoA which has two more carbon atoms than the initial compound.[2] By repeating this synthetic

process, fatty acids with longer hydrocarbon chains can be generated. The conversion from saturated to unsaturated fatty acids is achieved by desaturases, which takes away two hydrogen atoms from two adjacent carbon atoms at a time.

Not all fatty acids can be synthesized by humans. The two essential fatty acids, namely α-linolenic acid (omega-3) and linoleic acid (omega-6), are both polyunsaturated fatty acids and must be acquired via diet. The human body utilizes these two essential fatty acids to synthesize other important polyunsaturated fatty acids, including docosahexaenoic acid and arachidonic acid which play key roles in brain development and maintaining normal brain functions.[263-265] In addition, several omega-3 and 6 fatty acids have been suggested to interact with SNARE proteins, e.g. syntaxin, to promote membrane fusion and regulate exocytosis.[135, 266]

5.1.3 Cholesterol

Cholesterol is the main sterol in animal cells, but is barely present in prokaryotes. It is one of the major components of the cell membrane, constituting approximately 20% of the total lipid content.[259] In addition, the synthesis of steroid hormones utilizes cholesterol as a precursor. The structure of cholesterol consists of a hydrophilic hydroxyl group and a hydrophobic group made up of a steroid ring and a hydrocarbon chain. The amphiphilic property of cholesterol enables it to be incorporated among the phospholipids in the cell membrane. The interaction between cholesterol and the fatty acid tails of the phospholipids gives rise to one of the main functions of cholesterol, regulating membrane fluidity and permeability. The regulation varies at different temperatures. At high temperatures, cholesterol hinders the movement of the fatty acid tails of the phospholipids and thus, membrane fluidity decreases and the membrane is less permeable for small molecules. At lower temperatures, the effects are the opposite.

The role of cholesterol in regulating exocytosis is in part demonstrated via the formation of lipid rafts. Lipid rafts are microdomains of lipids within which cholesterol clusters with saturated phospholipids as well as sphingolipids.[267, 268] Unsaturated phospholipids, however, are typically not present in the lipid rafts. The function of lipid rafts is considered to be that they provide sites for interactions between certain lipids and exocytosis-related proteins, e.g. SNARE proteins.[269, 270] This suggests the possible role of cholesterol in regulating membrane fusion during exocytosis. In addition, similar to PE, cholesterol can also generate a negative curvature when present

in the cell membrane. The increased level of cholesterol induced by repetitive stimulation can thus assist in stabilizing the exocytotic fusion pore (**paper V**).

5.2 Mass Spectrometry Imaging (MSI)

MSI is a group of imaging techniques applied to obtain spatial information of molecules across a sample surface. In biological applications, the molecules to be analyzed can be peptides, proteins, lipids, biomarkers, etc. The entire sample surface can be divided into multiple small sections, called pixels. During MSI, an ion beam or a laser is typically used to ionize the sample surface pixel by pixel until the entire sample surface is covered. The ions generated during the ionization process from each pixel are separated and analyzed by a mass analyzer, which gives rise to a mass spectrum. By selecting a peak from the molecule of interest within each spectrum, the spatial distribution of this molecule across the sample surface can be obtained. Although MSI is thought to be a qualitative technique, relative quantification can be achieved as a change of the abundance of an analyzed molecule leads to a relative alteration of the peak intensity in the mass spectrum.

Matrix-assisted laser desorption ionization (MALDI) imaging is one of the most frequently used MSI techniques. In MALDI, a matrix is used to absorb energy from the laser in order to induce desorption and ionization of molecules from a sample surface. As the ionization method in MALDI is relatively soft, less fragmentation is induced and relatively large molecules can be detected. Thus, MALDI has become a powerful tool for protein analysis since its first application of biological samples.[271, 272] However, this also leads to a limitation of MALDI which is the detection of low-mass or small molecules. Another limitation is that the spatial resolution of MALDI (typically 10-50 μm) makes it hard to perform cellular analysis.[273] This limited spatial resolution is partly because of the size of the laser, the size of the matrix crystals, and the diffusion of molecules during sample preparation.[274] However, it is important to note that with certain approaches, MALDI can be carried out with better spatial resolution.[275, 276]

The general limitations of MALDI can be compensated by another MSI technique, secondary ion mass spectrometry (SIMS) imaging. Different from MALDI, SIMS utilizes a focused primary ion beam to generate secondary ions from a sample surface. The spatial resolution of SIMS falls into the range of micro- to nanometer scale. A variety of ion sources have been used as primary ion beams in SIMS, including liquid metals (e.g. Bi_3) and gas clusters (e.g. Ar and CO_2 with cluster size 500 - 4000). Due to the small sizes

of the liquid metal ion beams, it is possible to focus the beam to nanometer-sized spots on the sample surface to achieve high spatial resolution. Moreover, short pulses of several nanoseconds can be generated by the liquid metal ion beams. However, since the primary ion beams typically contain high energy density, the impact of the primary ions often damages the focused spots on the sample surface and results in the breaking of intact molecules into fragments, leading to loss of molecular information. The development of gas cluster ion beams overcomes this problem. Although lacking the high spatial resolution provided by the liquid metal ion beams, the energy per atom within the gas cluster ion beams is much lower and causes less damage upon reaching the sample surface.[277] Thus, less fragmentation is produced and more secondary ions can be generated, enabling the detection of larger as well as more intact molecules.[278]

SIMS can be operated under either static mode or dynamic mode. Static SIMS is typically utilized to obtain elemental and molecular information from a sample surface. It is performed with a pulsed primary ion beam that possesses a relatively low ion dose (less than 10^{13} ions/cm^2) and therefore, a very thin layer of the sample surface is impacted. This leads to less destruction as well as less fragmentation of the sample surface.[279] One example of static SIMS is the time-of-flight SIMS (ToF-SIMS), covered in more detail in the next section. Dynamic SIMS, in contrast, is more destructive and is used to obtain mainly elemental information. It employs a continuous primary ion beam with high ion dose to strike the sample surface, which causes severe fragmentation and enables the primary ions to travel deeper into the sample. The primary ion beams used for dynamic SIMS are typically monatomic ions such as Cs^+ or O^-, allowing the beams to focus on a submicron-sized region. Therefore, dynamic SIMS is capable of offering both high spatial resolution and depth information. An example of dynamic SIMS is the NanoSIMS, which not only provides a great spatial resolution (down to 50 nm), but also has good sensitivity and mass resolution.[280]

5.2.1 ToF-SIMS Imaging

ToF-SIMS is a MSI technique which separates secondary ions based on their different velocities in the ToF mass analyzer. It allows simultaneous detection and examination of all emitted secondary ions. A schematic of the ToF-SIMS process is illustrated in Figure 15. In ToF-SIMS, most instruments use a short pulsed primary ion beam to strike the sample surface to generate secondary ions. These secondary ions are then accelerated by an electric field to ensure all ions with same charge to possess the same kinetic energy. The velocity of each ion when traveling through the ToF mass analyzer is related

to its mass-to-charge ratio. Lighter ions travel faster and arrive at the detector earlier compared to heavier ions with the same charge. Therefore, the mass-to-charge ratios of different ions are reflected by their measured traveling time. However, when secondary ions with the same mass are emitted from the sample surface, their initial energies and emitted angles are not always the same.[279] Thus, the flight time of these ions from the sample surface to the detector can be different, which results in poor mass resolution. To improve this, an ion mirror, also called a reflectron, is often used.[281] The ion mirror utilizes an electrostatic field to reflect the secondary ions towards the detector. Ions with higher kinetic energy travel further into the ion mirror before being reflected, while lower energy ions travel a shorter distance. Thus all secondary ions with the same mass but possessing different initial kinetic energies reach the detector with a smaller distribution of time, leading to a better mass resolution.

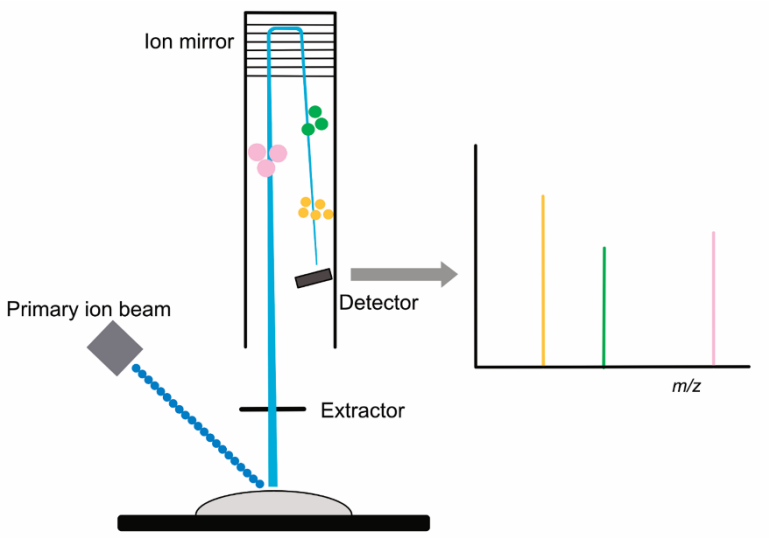

Figure 15. A schematic showing the principle of ToF-SIMS imaging. A primary ion beam is used to strike the sample surface to generate secondary ions, which are separated based on their *m/z* and subsequently detected.

The development of ToF-SIMS allows localization as well as identification of interested molecules, such as lipid species, within many biological systems. It has been applied to analyze lipidomics related to various diseases and disorders, such as cardiovascular disease, Alzheimer's disease, and brain injuries.[282-284] Lipid changes induced by cognition-related substances, including cocaine, MPH, and zinc have been investigated in

Drosophila brain using ToF-SIMS (**paper IV**) and a possible connection between lipid alteration and cognition has been suggested.[285, 286] In addition, the combination of electrochemical techniques and ToF-SIMS imaging has improved our understanding regarding the role of membrane lipids in regulating exocytosis (**paper V**).[287, 288]

Chapter 6. Summary of Papers

The work included in this thesis demonstrates the applications of several electrochemistry and imaging techniques to investigate the mechanism and regulation of exocytosis as well as the formation of plasticity in mammalian cell lines and the invertebrate *Drosophila melanogaster*. The combination of multiple techniques enables us to study exocytosis from different aspects and offers a more comprehensive view regarding this complex process of cellular communication.

In **paper I**, two electrochemical techniques, SCA and IVIEC, were applied with nanotip electrodes to study the effects of cocaine and MPH on exocytosis and vesicular content in PC12 cells. Cocaine and MPH are psychostimulants and affect neurotransmission similarly by blocking DAT to inhibit dopamine uptake. However, their influences on cognitive ability are the opposite with cocaine impairing cognition and MPH improving it. The results showed that although cocaine and MPH treatments both lead to decreases in vesicular transmitter content and exocytotic release, cocaine induces a higher degree of decrease in exocytosis compared to MPH. Thus, it is interesting that the release fraction is decreased upon cocaine treatment and increased after MPH treatment, both in a concentration-dependent manner. Peak analysis of SCA results suggest the formation of a less stable and perhaps smaller-sized fusion pore after cocaine treatment. MPH, on the other hand, does not affect the stability of the fusion pore. Mechanism regarding the actions of cocaine and MPH on neurotransmission is considered to involve several aspects, including DAT activity, actin activity, and membrane lipid structure. In addition, the differential alterations of release fraction caused by cocaine and MPH suggest a possible connection between release fraction and cognition.

In **paper II**, we combined SCA and IVIEC with Ca^{2+} imaging to examine the alteration of neurotransmission strength during the formation of activity–dependent plasticity. Single PC12 cells were repetitively stimulated six times with 2-min in between, and exocytosis was measured with disk electrodes. I found that the number of exocytotic release events is significantly reduced upon six repetitive stimuli, indicating a depletion of exocytotic machinery. However, the average amount of release per event increases, although not always significantly. To understand the mechanism underlying the decreased number of events, we examined the Ca^{2+} levels during the first and the sixth stimulation using Ca^{2+} imaging. The significant decrease of Ca^{2+} level upon repetitive stimuli correlates with the fewer number of release events

observed. Vesicular contents without stimulation, after three stimuli, and after six stimuli were quantified with IVIEC at nanotip electrodes. The IVIEC results indicate that repetitive stimuli induce a reduction in vesicular content. The release fraction, however, is enhanced after repetitive stimuli to almost full release. To explain these opposite changes, peak analysis of SCA data was carried out. The duration, especially the opening phase, of the fusion pore becomes longer upon repetitive stimuli, which is also confirmed by the analysis of pre-spike foot. Therefore, during repetitive stimuli, although fewer vesicles are undergoing fusion process and each vesicle stores lower amount of transmitters, the fusion pore from individual vesicles tends to stay open for a longer time to allow a higher fraction of transmitter storage to be released. This increase in the release fraction can be viewed as a way to compensate the loss of releasable vesicles and the reduced transmitter storage, thus a new paradigm of plasticity. In addition, the interplay between actin and dynamin as well as alteration of membrane lipid composition in the exocytotic active zones has been suggested to promote fusion pore stability during repetitive stimuli.

Paper III compares SCA measurements with nanotip and disk electrodes from PC12 cells to evaluate the use of nanotip electrodes for quantifying exocytosis. The average amounts of exocytotic release measured by these two electrodes show no difference, but the dynamics of release varies. Nanotip electrodes give on average a slightly higher and narrower spike compared to disk electrodes, indicating that the rate of exocytotic release is faster when measured by nanotip electrodes. To understand the possible reasons leading to this difference, simulations were applied to estimate the distance between the electrode and the exoyctotic fusion pore as well as the fusion pore size for both types of electrodes. When placing a nanotip electrode instead of a disk electrode on top of a cell, the distance from the electrode to the fusion pore becomes smaller, whereas the size of the fusion pore turns larger, leading to the observation of higher and narrower spikes. By comparing the molecular distributions for exocytotic release measured by these two electrodes, we found that the two distributions do not overlap. Since disk electrodes have a lower noise level, when setting the criteria for spike selection the same for both electrodes, disk electrodes give rise to the detection of more small release events compared to nanotip electrodes. By increasing the spike selection criteria for disk electrodes, the two distributions show no difference, demonstrating the importance of considering spike selection criteria when comparing data between different types of electrodes. Taken together all the results, we suggest the use of nanotip electrodes for measuring both SCA and IVIEC to make quantitative comparison between exocytosis and vesicular content.

Paper IV examines lipid alterations caused by zinc deficiency in the central brains of *Drosophila melanogaster* using ToF-SIMS imaging. Flies were fed with either normal fly food or zinc-chelated food throughout the entire life cycle. The average levels of zinc inside fly larvae, adult fly heads, and adult fly bodies were examined to confirm that zinc deficiency was successfully induced. By imaging the central fly brains, higher abundances of PC and PI were observed in flies with zinc deficiency. The alterations of PE species are more complex. PE with 34 carbons in the fatty acid tails has increased abundance in the zinc deficiency fly brains, whereas PE with 36 carbons in the fatty acid tails shows a lowered level. As a deficiency in zinc can lead to abnormal brain development and have negative impacts on cognition, lipid changes observed in this study might reflect a potential treatment target for cognitive impairment induced by zinc deficiency. In addition, zinc deficiency and cocaine treatment have similar effects on lipid composition, suggesting a possible link between cognitive impairment and the alterations of certain membrane lipids.

Paper V is a follow-up study of paper II. Here I attempted to investigate the effects of repetitive stimuli on membrane lipid composition in PC12 cells using ToF-SIMS imaging and correlate with the alterations observed in exocytosis. Cells were repetitively stimulated for three or six times and membrane lipids were imaged. The results were compared to cells without any stimulation. The abundance of PC drops significantly after three or six stimuli in comparison to no stimuli. The levels of PE, PI, and cholesterol are elevated after three stimuli and are elevated further more after six stimuli. By comparing to previous electrochemical data, I suggest a correlation between the alteration of membrane lipid composition and the stabilization of the exocytotic fusion pore following repetitive stimuli. The decrease of PC, cylindrical-shaped lipid, and increase of both PE and PI, conical-shaped lipids, after repetitive stimuli give rise to higher curvature of the cell membrane and the exocytotic fusion pore, a structure with high curvature, is suggested to be more stabilized. This stabilized fusion pore can therefore release a higher fraction of stored vesicular transmitters.

In **paper VI**, a technique to simultaneously monitor vesicular transmitter storage and vesicular release with two nanotip electrodes in and at the same PC12 cell was developed. By inserting one nanotip electrode inside a cell to perform IVIEC and placing the other nanotip electrode on top of the same cell to do SCA, a direct comparison between vesicular storage and vesicular release before as well as during and after the stimulation can be achieved. In addition, the real-time changes of vesicular transmitter storage in response to a chemical stimulation can be directly followed. Even though

chemical stimulation induces a significant depletion in average vesicular storage, it is still much higher than the measured vesicular release, again demonstrating partial release as the dominant mode of exocytosis. Under normal conditions, not all vesicles respond to a stimulation to release their transmitter loads. Using the IVIEC data, I made a preliminary estimation that approximately 21% of all vesicles in the cell undergo exocytosis. Examining the IVIEC results after stimulation with a time interval of 5 s, an increase followed by a decrease is observed in vesicular storage between 5 and 20 s post-stimulation. Assuming the existence of multiple subpools of vesicles inside the cell, this increase is suggested to be due to a replenishment of the releasable pool with a subpool of more mature vesicles which store a higher amount of transmitters. Alternatively, if all vesicles within the cell belong to one pool and are more or less homogeneous, the observed increase might represent a transient transmitter loading of some unreleased vesicles to compensate for the loss of releasable vesicles upon stimulation, a rapid form of plasticity. The decrease observed after the increase is suggested to come from the previous partially released vesicles, which, with time, are partially refilled. In addition, the effect of enhanced vesicular transmitter loading caused by L-DOPA incubation on the dynamic alteration of vesicular transmitter storage was also investigated. Interestingly, the average vesicular storage is not reduced after stimulation following L-DOPA incubation. Moreover, no significant alterations of vesicular storage can be observed after dividing the post-stimulation IVIEC results into a time interval of 5 s. These data indicate that enhanced vesicular loading might be adequate to maintain the neurotransmission strength and thus, the formation of rapid plasticity is not observed after L-DOPA. The two-electrode technique allows the comparison between vesicular storage and vesicular release from the same cell to provide insights into stimulation-induced plasticity.

Chapter 7. Concluding Remarks and Future Outlook

The main focus of this thesis work has been to develop and apply methods to study mechanism and regulatory factors governing exocytosis, the major form of cellular communication, in an effort to enhance our understanding of the vital role of cellular communication in the survival of multicellular organisms. Exocytosis is a highly complicated process and studying all aspects of exocytosis at the same time is difficult. Therefore, studies are typically focused on one or a few aspects of exocytosis. Secretory vesicles can be mainly divided into two categories, SSVs and LDCVs. These differ in size and inner structure and have some differences regarding the type of neurotransmitters they store. Several steps are involved in exocytosis, including docking, priming, and fusion, and many proteins are important to maintain the proper functioning of exocytosis. Priming and the subsequent fusion of a vesicle require the coordination of a group of proteins including the SNARE proteins, the SM proteins and synaptotagmin. The interplay between actin and dynamin is considered to, at least in part, govern the dynamic activities of the fusion pore. In addition to proteins, Ca^{2+} and membrane lipids also play key roles in exocytosis. Elevation of the cytosolic Ca^{2+} level, either by rapid Ca^{2+} influx or the release of intracellular Ca^{2+}, is necessary for the initial opening of fusion pore. The reorganization of membrane lipids towards an increased curvature is another essential factor promoting the formation of the fusion pore.

Methodologies used for studying exocytosis and membrane lipids have been described in this thesis. Electrochemical techniques can either offer high temporal resolution to follow and quantify exocytosis from single vesicles, or measure the release and uptake of specific signaling molecules. Electrophysiological techniques, on the other hand, can be used to monitor ion channel activities and vesicle fusion. By labeling interested molecules, fluorescence imaging can be employed to visualize vesicle movements and protein distributions. The use of MSI offers spatial information regarding membrane lipids and allows relative quantification.

The papers included in this thesis employ several methodologies to investigate exocytosis and lipid alteration related to plasticity and cognition in several model systems. The work supports partial release as the dominant mode of exocytosis, at least in the model systems studied. I have shown that the release fraction can be altered via pharmacological treatment or cellular activity, and might be involved in the formation of plasticity as well as cognitive ability. However, most of the work was done in neuroendocrine cells

and focused on exocytosis of catecholamines from LDCVs. Investigating the release of other types of neurotransmitters, the release from SSVs, and exocytosis from human neurons might offer more insights into the different modes of exocytosis. Moreover, a real-time combination of different methodologies, such as electrochemical techniques and high-resolution fluorescence imaging, might shed light on a broader perspective regarding the mechanism of cellular communication.

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