Thesis for the Degree of Doctor of Philosophy
in the Natural Sciences

Electrochemical and Microscopic Analysis of Chemical Signalling in Biological Systems

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Chemical Signalling in Biological Systems

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Cover: Illustration by the author showing vesicles and an electrode

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Abstract

Cellular communication is a prerequisite in multicellular organisms in order to survive. Many times, this communication occurs through the highly controlled and regulated release of chemical signals through a process known as exocytosis. This process consists of organelles known as vesicles fusing with the plasma membrane to release the content inside to the extracellular space. Although the basic underpinnings of this process are known, exactly how it is regulated is still largely unknown.

This thesis covers studies performed on mammalian cell lines and invertebrate neurons with the aim to further understand regulation of exocytosis. Several complementary methodologies have been utilised to study this regulation from different points of view. The electrochemical technique of amperometry has the benefit of being able to track the exocytotic process with high temporal resolution and makes it possible to quantify both how many molecules are stored in a vesicle as well as how many are released. Several imaging methods, such as fluorescence, electron microscopy and mass spectrometry, provided high quality spatial information to complement the electrochemical techniques.

The papers included in this thesis have involved studies of how exocytosis is regulated in PC12 and chromaffin cells, along with how it is affected by pharmacological treatments as well as more intrinsic factors such as the secretory activity of the cell. In paper I, storage and release of dopamine was determined both using amperometry and imaging mass spectrometry. In paper II, the drug tamoxifen was observed to regulate both transmitter storage and release. ATP also was shown to regulate transmitter storage and release as demonstrated in paper III. Paper IV further provides an additional role for ATP as regulating vesicle content in combination with norepinephrine. Repetitive stimuli regulate exocytosis by causing cells to release larger fractions of their stored content as seen in paper V. In addition, a method previously developed in our group, intracellular electrochemical vesicle cytometry, was adapted and applied to measurements of vesicle content in the more complex and considerably smaller biological model system of the *Drosophila* neuromuscular junction in paper VI.

In the biological model systems studied here, exocytosis appears to most often occur through vesicle fusion and closure that only allows some of the vesicular content to

escape. By only partially releasing the molecules stored inside vesicles, the chemical signalling can be regulated and adapted. The presence of this feature opens new possible drug targets in order to medically alter dysfunctional chemical signalling in diseases as well as a possible key to understand how memories are formed.

Sammanfattning på svenska

Att celler kan kommunicera med varandra är ett krav för att flercelliga organismer ska överleva. Denna kommunikationen sker ofta genom en högst kontrollerad process som kallas exocytos som möjliggör att celler kan skicka kemiska signaler. Under exocytos går organeller som kallas vesiklar ihop med cellmembranet för att utsöndra sitt molekylära innehåll. Trots att de grundläggande aspekterna av exocytos är kända är det inte klart exakt hur denna process regleras.

I denna avhandling beskrivs studier gjorda på celler från däggdjur och nervceller från bananflugor med syfte att vidare förstå reglering av exocytos. Analytiska tekniker som kompletterar varandra har använts för att studera denna reglering från flera synvinklar. En elektrokemisk metod som kallas amperometri har fördelen att kunna följa det exocytotiska förloppet med hög tidsmässig precision och gör det möjligt att kvantifiera både hur många molekyler som finns i en vesikel och hur många som utsöndras. Metoder för att avbilda celler har också använts. Dessa inkluderar fluorescens, elektronmikroskopi och masspektrometri vilka kompletterar amperometrin.

Artiklarna i denna avhandlingen redogör för hur reglering av exocytos studerats i PC12 och kromaffin celler. Fokus ligger på hur regleringen påverkas av både farmakologiska preparat och intrinsiska faktorer så som cellens aktivitet. Utöver det beskriver en av artiklarna hur en metod som tidigare utvecklats i vår grupp anpassas för att kunna mäta antalet molekyler i bananflugors nervceller.

I alla artiklar verkar exocytos ske genom att en vesikel snabbt öppnas och stängs innan allt innehåll avgetts. Genom att endast utsöndra en del av innehållet i en vesikel kan den kemiska signalen regleras och anpassas efter behov. I framtiden kan detta göra det möjligt att hitta nya mål för läkemedel som justerar felaktig cellkommunikation i sjukdomar. Utöver det kan denna process vara en viktig del för att förstå de första molekylära stegen för hur minnen skapas.

List of publications and contribution report

I. **Nano Secondary Ion Mass Spectrometry Imaging of Dopamine Distribution Across Nanometer Vesicles**
Lovric, J., Dunevall, J., **Larsson, A**., Ren, L., Andersson, S., Meibom, A., Malmberg, P., Kurczy, M. E., Ewing, A. G. *ACS Nano* (2017), 11(4), 3446-3455

Performed and analysed the single cell amperometry data, interpreted and discussed the data with the other authors, and participated in writing and editing the manuscript.

II. **Anticancer Drug Tamoxifen Affects Catecholamine Transmitter Release and Storage from Single Cells**
Taleat, Z., **Larsson, A.,** Ewing, A. G *ACS Chemical Neuroscience* (2019) 10(4), 2060-2069

Designed, performed and analysed the fluorescence imaging experiments, interpreted and discussed the data with the other authors, and participated in writing and editing the manuscript.

III. **Extracellular ATP Regulated the Vesicular Pore Opening in Chromaffin Cells and Increases the Fraction Released During Individual Exocytosis Events**
Larsson, A.†, Majdi, S.†, Najafinobar, N., Borges, R., Ewing, A. G. (2019) *ACS Chemical Neuroscience* 10(5) 2459-2466

Participated in designing and performing the experiments as well as analysed and interpreted the data with S. Majdi, discussed the results, and participated in writing and editing the manuscript.

IV. The Vesicular Transmitter Content in Chromaffin Cells can be Regulated via Extracellular ATP

Larsson, A.†, Majdi, S.†, Borges, R., Ewing, A. G. Submitted

Participated in designing and performing the experiments as well as analysed and interpreted the data with S. Majdi, discussed the results, and participated in writing and editing the manuscript.

V. Plasticity in Exocytosis Revealed Through the effects of Repetitive Stimuli Affect the Content of Nanometer Vesicles and the Fraction of Transmitter Released

Gu, C., **Larsson, A**., Ewing, A. G. *PNAS*, In press

Participated in designing and performing the fluorescence imaging experiments, discussed the data, participated in writing and editing the manuscript.

VI. Exocytosis Events at a Living Neuron are Sub-Quantal and Complex

Larsson, A.†, Majdi, S.†, Oleinick, A., Svir, I., Dunevall, J., Amatore, C., Ewing, A. G. Manuscript in preparation

Performed the electrochemical experiments, analysed the data, interpreted and discussed the data, and wrote and edited the manuscript with S. Majdi and A. Ewing.

† These authors contributed equally

Related papers not included in the thesis

Vesicle Impact Electrochemical Cytometry Compared to Amperometric Exocytosis Measurements, Dunevall, J., Majdi, S., Larsson, A., Ewing, A. *Current Opinion in Electrochemistry* (2017) 5(1), 85-91

Electrochemistry in and of the Fly Brain, Majdi, S., Larsson, A., Hoang Philipsen, M., Ewing, A. G. *Electroanalysis* (2018) 30(6), 999-1010

Ex Situ **and** ***In Situ*** **Structural Studies of Large Dense Core Vesicles using Electron Microscopy**, Larsson, A., Majdi, S., Zabeo, D., Höög, J., Ewing, A. G. Manuscript in preparation

Abbreviations

ATP	adenosine triphosphate
ADP	adenosine diphosphate
CE	capillary electrophoresis
CLEM	correlated light and electron microscopy
CNS	central nervous system
CV	cyclic voltammetry
EM	electron microscopy
ER	endoplasmic reticulum
ET	electron tomography
FFN	false fluorescent neurotransmitter
FSCV	fast scan cyclic voltammetry
GFP	green fluorescent protein
HPLC	high pressure liquid chromatography
IMS	imaging mass spectrometry
IVIEC	intracellular vesicle impact electrochemical cytometry
LDCV	large dense core vesicle
MALDI	matrix assisted laser desorption ionisation
MS	mass spectrometry
NGF	neuronal growth factor
NMJ	neuromuscular junction
SCA	single cell amperometry
SIMS	secondary ion mass spectrometry
SNARE	SNAP receptor
SV	synaptic vesicle
Tdc	tyrosine decarboxylase
TEM	transmission electron microscopy
TGN	trans-Golgi network
TIRFM	total internal reflection microscopy
UAS	upstream activating sequence

VIEC	vesicle impact electrochemical cytometry
VMAT	vesicular monoamine transporter
VNUT	vesicular nucleotide transporter

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Chapter 1. The Basics of Cell Communication

In all multicellular organisms, communication between cells is vital for survival and dysfunctional communication can result in severe pathologies such as neurodegenerative diseases. Studying the functionality and regulation of cellular communication is therefore crucial for understanding and hopefully ameliorating these pathologies. This chapter introduces cellular communication and how a signal is propagated from one cell to another. Four key transmitters that have been the focus for the work covered in this thesis are also described further with an emphasis on their biological importance.

1.1 Cell-to-cell communication

The human body consists of an unfathomably complex network of cells. These cells come in different shapes and functions but all work toward a common goal, survival. For this to occur, the cells need to be able to respond both to external and internal changes in the environment. Although all cells need to communicate in one way or another, neurons are highly specialised in collecting, "processing", and sending forward signals in the central and peripheral nervous systems. This is done through a combination of electrical and chemical steps that are described further down in 1.2 Intracellular propagation of chemical signals. A basic illustration of a neuron and its synaptic connection to a neighbouring cell is shown in Figure 1.1.

The dendrites are processes that gather signals from other neurons via receptors and move these signals along towards the cell body, or soma. Inside the soma most of the typical eukaryote organelles are present: mitochondria, Golgi apparatus, nuclei, ribosomes and many more. The soma is also the starting point of the axon, a long process responsible for sending forward signals received by the cell. It is between the end of the axon and the receiving part of another cell that the synapse is formed, encompassing the release of chemical transmitters from the so-called active zone across the synaptic cleft which is ~20-30 nm wide.[1,2] While this is the typical process and signalling direction of a neuron, there are exceptions. For instance, release has been known to occur from the soma or the dendrites.[3] In some cases there is no synaptic cleft

at all and the forwarding of the signal occurs through so-called gap junctions, proteinic immediate connections between two cells.[4] The initiation of release might also be independent of an incoming signal, so-called spontaneous release.[5,6]

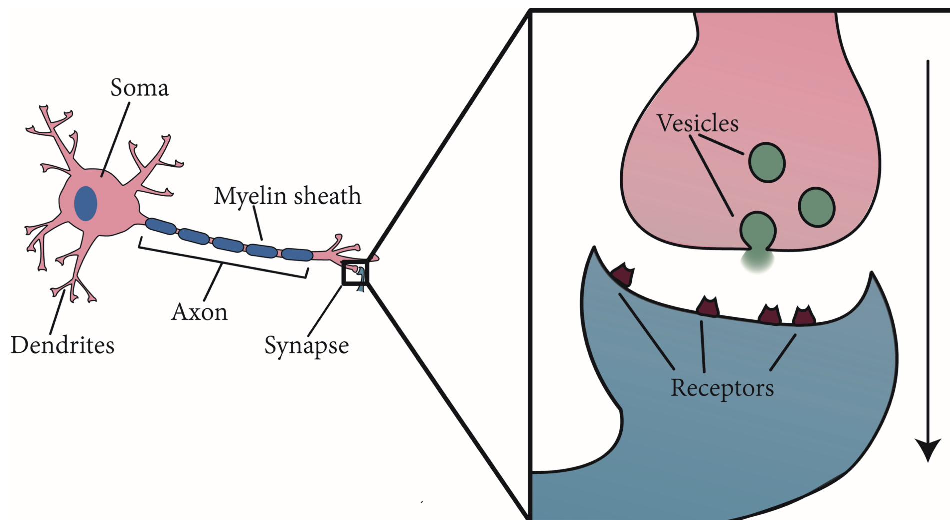

Figure 1.1 Schematic of a neuron showing some vital structures for cellular communication. The enlargement shows how a chemical signal in propagated in the synapse. Vesicles release neurotransmitters into the synaptic cleft where they diffuse to receptors on the receiving neuron.

Although a neuron is the quintessential model for illustrating highly specialised cellular communication, cell types of other origin and function also exhibit structural adaptations to streamline signal propagation.[7] As an example, pancreatic β-cells that are part of the endocrine system target the release of insulin towards nearby blood vessels. This is potentially to facilitate the transport of insulin to target tissue, a process that would have been slowed down through non-specific release all around the cell.[8]

Just as different cells have different functions, their communication is adapted to specific purposes. Within the brain and the central nervous system (CNS), neurons need to communicate quickly. Thus, fast release and resetting of the synaptic cleft is vital. Small, rapidly diffusing molecules are typically released and expression of plasma membrane transporters make sure the cleft is not left continuously in the "on" state by clearing away superfluous transmitters.[9,10] However, out in the body, release from the endocrine system is not as dependent on time but might instead need to convey signals

across larger distances such as between the adrenal gland and the heart. This requires a different approach to communication, where potentially larger quantities of the transmitters are released over a longer time period as well as additional molecules such as peptides.[7,11–13] This is what occurs in e.g. chromaffin cells in the adrenal gland (3.2 Chromaffin cells). Nevertheless, one aspect that is crucial for cellular communication is plasticity. The ability to adapt the communication between cells is important for the organism to not only respond to the environment, but also to learn and develop.[14–17] These adaptations can take place by changing the structure of how the cell connects (i.e. more dendrites or synapses), by adjusting the strength of the released signal (presynaptic plasticity), or by making the receiving cell more sensitive (postsynaptic plasticity).[15,18–20] Many of the details of how cells regulate this signalling strength and the mechanisms plasticity originates from are unknown, but critically important, making it an interesting area to study. The large number of proteins and molecules needed to act in unison in order to propagate a signal between cells creates further complexity and will be discussed further on in this chapter and the next (Chapter 2. Exocytosis).

1.2 Intracellular propagation of chemical signals

The initiation of an intracellular chemical signal can be different in form and origin depending on the cell type. In neurons, synaptic transmission typically starts when an electrical signal is propagated along the cell. This signal is caused by differences in the concentration of potassium and sodium ions across the plasma membrane that rapidly fluctuate when specific voltage gated ion channels open in synchrony.[21] In other secretory cells such as those in the endocrine system, initiation can instead be from a chemical input. Many cells express receptors on their plasma membrane that respond to the presence of a certain molecule in the extracellular solution.[22,23] The receptor then mediates the response further intracellularly before the signal is propagated.

Secretory cells such as neurons, endocrine cells and inflammatory cells all use increased cytosolic calcium as a trigger for chemical signalling.[24–26] Due to the importance of calcium as an intracellular messenger, the cytosolic levels are typically tightly regulated. For instance, the cytosolic calcium levels are kept four orders of magnitude lower than in the extracellular solution in vertebrates. However, when an

electrical signal reaches the active zone in a synapse, voltage gated calcium channels open to allow a rapid influx of calcium.[21] As enough channels open, the concentration of calcium reaches sufficient levels to trigger the release of molecules from vesicles.

In addition, cells also keep internal stores that can liberate calcium when needed. Some of the organelles involved in calcium storage are the endoplasmic reticulum (ER), mitochondria, the nuclear envelope, as well as secretory vesicles.[27,28] The detailed effects of internal calcium stores on chemical signalling through vesicular release are not known and might be complicated because of a behavioural duality. Calcium storage organelles have been observed to both uptake calcium in a buffering manner, as well as release calcium to further amplify an already present calcium signal.[27,28]

Although an increase of cytosolic calcium is necessary for cellular communication in many cell types, too much calcium over a longer period of time has adverse effects on cell viability. This can cause the phenomenon known as excitotoxicity, where a cell is stimulated too harshly, resulting in severe cell damage or even death. Elevated calcium levels are believed to be one link in the chain between hyperstimulation and cell death.[29] The distinctly different effects of low, medium and high calcium levels also illustrates how regulation in cellular communication is really a delicate balance as many of the molecules involved in these processes can be both vital for basic functionality and detrimental if present at the wrong place in the wrong time or at the wrong concentration.

1.3 Types of transmitters

The main secreted molecule in cellular communication is typically known as a transmitter, or neurotransmitter, but there are also other molecules that in some way work to adjust and modulate the chemical signal. These are known as neuromodulators and work through a number mechanisms of action that either enhance or diminish the chemical signal.[30]

Within this work, four transmitters are the major focus of study and will be described in larger detail (Fig. 1.2). They are all electroactive and can thus be analysed using electrochemical techniques described later (Chapter 4. Electrochemical Analysis). However, there are is a large variety of transmitters and each have their own specific

chemical and biological roles with regard to what physiological responses they influence and how they are regulated. Despite this, the processes described above and further in Chapter 2 are highly conserved between species as well as different transmitters.[7,21] This means that even though a specific transmitter is studied, conclusions are potentially relevant for others after adaptation.

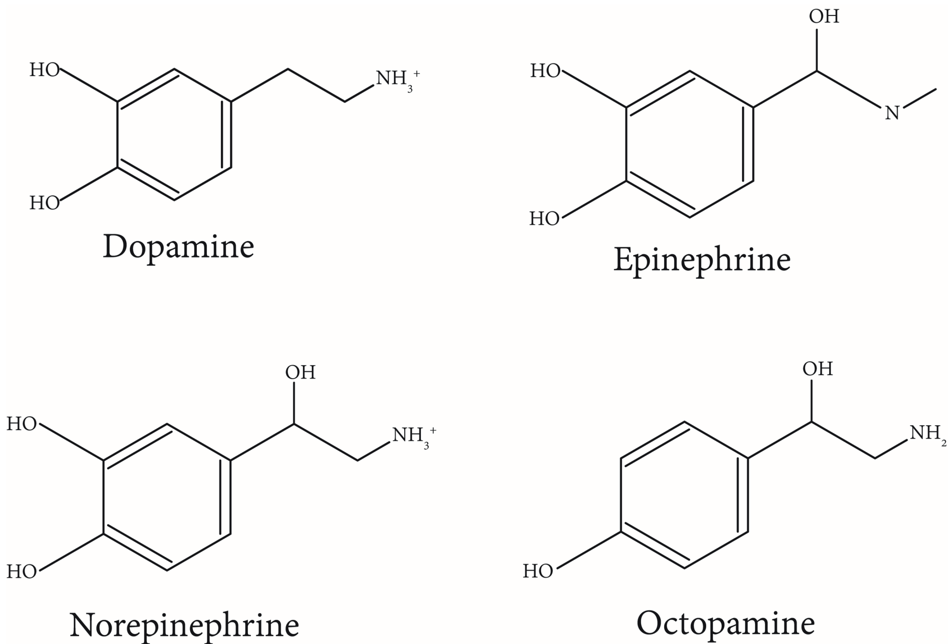

Figure 1.2 Chemical structures of the four main transmitters studied within the scope of this thesis.

One of the neurotransmitters in focus in this work has been dopamine, a biogenic amine of high importance in both mammalian and invertebrate systems. In humans, it is mainly used and synthesized in the substantia nigra and ventral tegmental areas of the brain, but the presence of dopaminergic receptors in a wide range of human tissue such as kidneys, heart, and pancreas hints of a broader functionality.[31–33] Learning, memory, sleep, attention, reward, and motor control are a few of the known features where dopamine plays a part.[34,35] Due to the distribution of dopamine and its many targets, it is not surprising that dysfunction in dopamine signalling gives rise to a plethora of diseases. Among those diseases where dopamine has a putative role are Parkinson’s disease, addiction, ADHD, and some types of psychosis.[36–39]

Two other transmitters of interest are epinephrine and norepinephrine. In contrast to dopamine, these are mostly known for their activity outside the brain. The main site of synthesis is in the medulla of the adrenal gland, and they are released when the gland is activated through the splanchnic nerve. As some epinephrine and norepinephrine is stored and released from large dense core vesicles, their release is also concurrent with release of peptides such as catestatin and secretoneurin.[40–42] Normally, epinephrine and norepinephrine are responsible for maintaining heart rate and blood pressure, but also gives rise to the so-called "fight-or-flight" response where a stressor causes release of epinephrine and norepinephrine to prime the individual to deal with the threatening situation through, for example, an increased heart rate and vasodilation in the muscles among other things. However, these transmitters have also been implicated as part of the pathophysiology of depression and hypertension.[43,44]

In invertebrate systems, octopamine has usually been ascribed to have a similar role to that of norepinephrine in humans. Octopamine is, like the other transmitters, electroactive and can thus be studied with ease using electrochemical methods.[45] In *Drosophila,* octopamine plays an important role for movement as shown in studies of larvae where genetically induced dysfunctional octopamine metabolism resulted in dysfunctional motor control.[46] Despite the fact that no physiological functions have been definitely determined for the low amounts of octopamine discovered in vertebrates, studies have implicated a neuromodulatory role of octopamine along with other amines present at low levels in the CNS.[47] The similarities of octopamine and vertebrate transmitters and its abundance in the well-established model organism *Drosophila,* causes continued scientific interest in this catecholamine.

Although these represent only four out of a large variety of transmitters available, the transmitters described above are involved in a broad range of bodily functions and diseases suggesting that regulation of chemical signalling is highly interesting. To delve deeper into this regulation, the next chapter will describe in more detail transmitter storage and release through the process of exocytosis.

Chapter 2. Exocytosis

2.1 Vesicles

The cellular organelles responsible for carrying and releasing neurotransmitters and other signalling substances are called vesicles. These vesicles can be roughly divided into two main types based on their size and what is contained inside their membrane. Synaptic vesicles (SV) are typically the smaller type and appear clear in negative stain transmission electron microscopy (TEM) as they do not contain a substantial amount of internal proteins.[48] This type of vesicle is commonly present in the CNS.[49] The other type is called large dense core vesicles (LDCV) and are, as indicated by the name, considerably larger and in negative stain TEM, an electron dense core is visible. This core is mainly made up of a mixture of proteins from a family called the chromogranins.[50,51]

Studies have shown that chromogranins bind to ATP and several neurotransmitters such as dopamine, norepinephrine, epinephrine, and serotonin, leading to the belief that the purpose of the dense core is to lower the osmotic pressure by binding solutes in the vesicle.[52,53] This lowered osmotic pressure is crucial as the high concentration of solutes otherwise would break the vesicle membrane.[54,55] In addition, the binding effect of the chromogranins contributes to how quickly the transmitters escape the interior of the vesicles once exocytosis is in progress. Studies of exocytosis where one or two chromogranin proteins have been knocked-out show that transmitter release both contains fewer numbers of molecules and has different release kinetics compared to wild type vesicles.[55–57] Other studies have also suggested the idea that vesicles contain two compartments in which the transmitters are distributed with one fast compartment, where the transmitters are soluble and freely diffusing, and one slow compartment, where the transmitters are bound or otherwise hindered to freely diffuse.[58] The compartment that dominates then affects how fast the transmitters can be released upon a stimulus and the resulting response.

LDCVs are present in a number of places in biological systems: CNS, neuromuscular junctions, insulin beta cells, and adrenal cells.[59–61] The differences

between LDCVs and SVs can be clearly visualized in the neuromuscular junction of *Drosophila* larva, where both SVs and LDCVs are present (Fig. 2.1A).

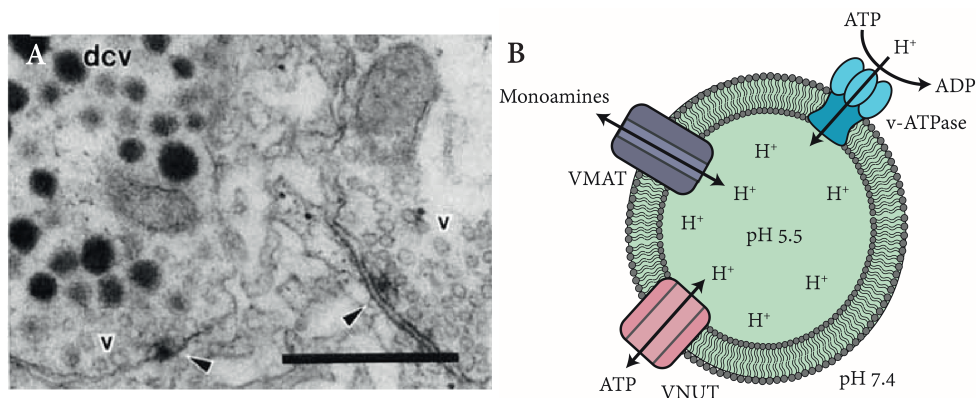

Figure 2.1 A. TEM of a *Drosophila* larva NMJ showing the presence of both clear synaptic vesicles (v) and large dense core vesicles (dcv). Arrows point to clusters of synaptic vesicles. Scale bar is 0.5 μm. Reproduced with permission from ref 62. B. A schematic of a secretory vesicle illustrating some features and protein functions necessary for secretory vesicle maintenance.

Several membrane proteins are also present on the transmitter carrying vesicles. One of these is the so-called vesicular H^+-ATPase. This protein is responsible for acidifying the vesicles by utilizing adenosine triphosphate (ATP) as an energy source to pump H^+ inside.[63] The result is a pH of ~5.5 compared to 7.4 as is common in the cytosol and extracellular space. The proton pumping also leads to a transmembrane charge due to the differences in ion concentrations. This difference in pH and membrane charge is in turn used by another protein, the vesicular monoamine transporter (VMAT).[64] By transporting H^+ out of the vesicle while also moving a monoamine such as dopamine into the vesicle, the accumulated concentration of transmitter can be as high as 0.5-1 M.[65]

Another transport protein is known as the vesicular nucleotide transporter (VNUT). The existence of a protein that could transport nucleotides into vesicles was long anticipated and only fairly recently discovered.[66] This protein is related to other transporters and also utilizes the membrane potential as a source of energy. The importance of this transporter can be related to the previously mentioned binding effect

of ATP, chromogranins and the transmitters for lowering the osmotic pressure. In addition, ATP is a transmitter or neuromodulator in its own right, thereby requiring the same regulatory loading and release as the more traditionally known transmitters.[67] A schematic of the proteins mentioned and their roles in loading and maintaining the functional status of the vesicle is shown in Fig 2.1B.

2.2 The exocytotic pathway

The typical life cycle of a vesicle is shown in Figure 2.2, and both types of previously mentioned vesicles have their origin in the endoplasmic reticulum (ER) and the trans-Golgi network (TGN) where they are synthesized and LDCVs are loaded with the dense core. After biogenesis, the vesicles are transported to the site of release, whether that is a synapse or other specialized area of the cell. The vesicles are docked by the plasma membrane and prepared or "primed" to release their content. The distinction and definition of these two steps is somewhat debated in the literature and it is not clear if this linear pathway of vesicle preparation is needed for functional release to occur.[68] However, docking has typically been seen as the step where the vesicle is bound and held at the active site in close proximity to the plasma membrane. This is most likely through protein interactions. After this step the vesicle is made trigger-ready by an ATP-dependent process known as priming.[69]

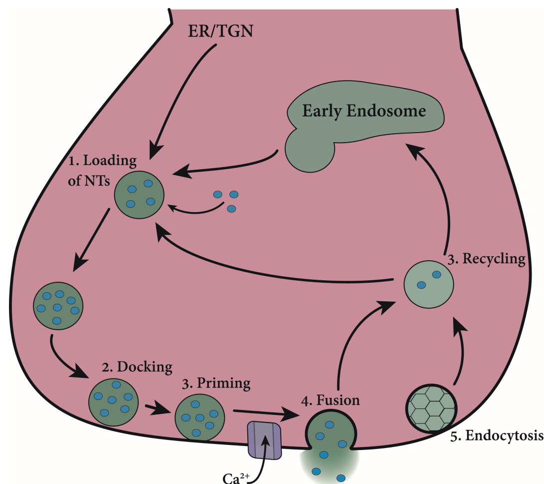

Figure 2.2. The life cycle of a secretory vesicle in a synaptic terminal illustrating localization and key steps for the release and reuse of vesicles after exocytosis. Blue dots represent neurotransmitters (NTs).

Once the vesicle has been primed, a signal of increasing cytosolic Ca^{2+} causes the vesicular and plasma membranes to fuse (1.2 Intracellular propagation of chemical signals). This occurs as the calcium ions cause the so-called SNARE complex to undergo a conformational change and bring the vesicle and cell membranes close enough together to fuse.[70] Exactly how calcium induces the change is unclear but potentially the protein synaptotagmin binds calcium and mediates the change to the SNARE complex.[71] The SNARE complex itself consists of three different membrane proteins that all share a similar motif, the SNARE motif.[72] Two of the proteins, syntaxin and SNAP-25, are anchored in the plasma membrane of the cell or soluble in the cytosol and the third, synaptobrevin, is attached to the vesicular membrane (Fig. 2.3A). The complex formed by these proteins is typically associated when the vesicle is near the membrane, but after the increase in calcium the proteins interlock in a zipper-like motion forcing the two membranes close enough together to form a pore.[73] A closer look on the complex formed by these proteins is shown in Fig dynamin are thought to regulate the process of opening and closing the pore.[74–76]

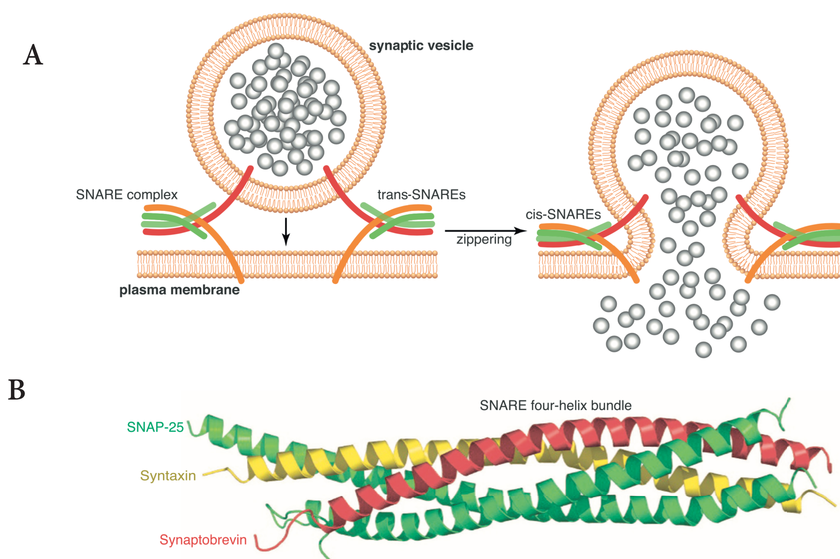

Figure 2.3 A. Schematic of how SNARE complex zippering results in vesicle fusion and release of transmitters. B. The structure of the zippered SNARE complex. Reproduced with permission from ref 77.

As the pore opens, the content inside the vesicle escapes and diffuses along the concentration gradient into the synaptic cleft or the blood stream. The molecules reaching the target cell, e.g. a postsynaptic neuron, bind to receptors which in turn open new ion channels, starting the transmission process over again towards another cell. For cellular signalling to function properly, the released transmitters also need to be cleared from their target as to not leave the switch constantly on (or off in case the bound transmitter has an inhibitory effect). The reuptake or breakdown of transmitters is performed by specific transporters and enzymes on the membrane to bring the extracellular transmitter levels back to normal, leaving the system ready for another round of synaptic transmission.

2.3 Regulation of exocytosis

There has been some debate of what exactly happens to the fusion pore after the initial SNARE facilitated opening.[78] Initially, it was thought that a pore formed and became increasingly larger until the whole vesicle eventually collapsed and combined with the plasma membrane. This mode of release is hereafter referred to as "full release" as the entire content of the vesicle is expelled to the extracellular space. However, continued research found evidence that this is not always the case and that the fusion pore can close before the vesicle collapses. There are a number of variations of this mode depending on how long the pore stays open and how much of the content is released.[78] Some of these modes are shown in Figure 2.3. For instance, in the kiss-and-run mode a small pore is only briefly open, allowing a minute amount of transmitter to escape the vesicle. Whereas in the mode herein called partial release, the pore does extend to some degree and stays open for longer for a larger amount of transmitter to be released.

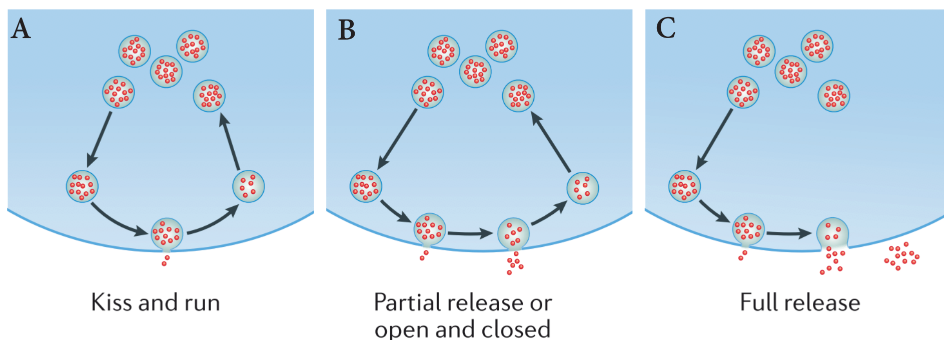

Figure 2.3 Exocytosis can occur though different modes: a, kiss-and-run, b, partial release, or c, full release. Note how the mode of exocytosis regulates the amount of transmitter being released. Reproduced with permission from ref 79.

Interestingly, partial release could be one of the features that allow cells to be plastic and modify their behaviour based on external or internal demands. Some research suggests that partial release is the most dominant mode of exocytosis and that full release only occurs sporadically or when the vesicle is damaged and should not be returned to the release site but completely broken down and recycled.[80,81] Which mode is dominant has been debated for quite some time and could potentially depend on a number of factors such as the cell type, stimulation strength and type, previous activity in the cell, or pharmacological treatment.[76,82,83] Through recent advances in methodology, both the amount of released transmitters as well as the total content inside vesicles can be measured and compared.[84,85] The fraction of released transmitters then provides quantitative insight to the extent of partial release and how it is affected by varying conditions such as activity (**paper V**) or pharmacological treatment (**papers II, III, and IV**).[86–88] Perhaps further studies on the regulatory role of partial release and the fraction of released transmitters will provide even more evidence into the mechanisms behind these phenomena as well as their importance in cellular communication.

Another aspect that could be part in exocytotic regulation is the speed or temporal regulation of the release events. Actin and dynamin have already been mentioned as potential proteins involved but also the rigidity of the membranes would affect the size and speed of the fusion pore opening and closing and thereby also the speed with which transmitters can exit the vesicle.[74,87,89] Other proteins that have been

suggested to regulate exocytosis are the aforementioned chromogranins that fill the inside of LDCVs. Transmitters bound to these proteins or soluble transmitters could be delayed in their release due to a slower diffusion out to the extracellular space.[55,90]

Besides these subtler regulatory mechanisms, exocytosis is also largely a numbers game. The amount of transmitter inside a vesicle and the number of vesicles available for release can of course also be used as regulatory mechanisms for a cell to enhance or decrease the outgoing signal.[91,92] Given the amount of knowledge still missing concerning the regulation of exocytosis and its implications in fields such as neuroscience and metabolic diseases, continued and future studies are required. While the sheer amount of regulatory mechanisms can be daunting, it also demonstrates the essential nature of the process. Cellular communication through exocytosis is too important to go unregulated, hence there appears to be a large number of mechanisms for controlling how, where and when transmitters are being released.

Due to this complexity, studying exocytosis and its regulation is a formidable task. Strategies are needed in order to dissect specific problems and questions into smaller, more manageable components. One way of simplifying, and to some degree control, the parameters in a study is by using biological model systems, the topic of chapter 3.

Chapter 3. Biological Model Systems

Although the human brain is often seen as the front figure for the field of cellular communication and exocytosis, it is also the most intricate and poorly understood organ we know of. Studies of the underlaying molecular mechanisms and regulatory processes of exocytosis can be hard to accomplish or interpret. One solution to this issue is to deconstruct the scientific question and to use simpler biological model systems. For example, animal models such as rats, mice and invertebrates or simple cell cultures containing only one type of cell are commonly used. In this chapter, the focus will be on models studied in the papers covered in this thesis: PC12 cells, chromaffin cells, and *Drosophila melanogaster*. The background and relevance of these models is addressed in the context of their use for studying cellular communication.

3.1 PC12 cells

In order to study exocytosis and cellular communication in a simple and contained system, isolated cells or cell lines are often used. One of these cell lines is called PC12, and was first isolated and cultured in 1976 in the lab of Lloyd Greene.[93] These cells originated from a rat pheochromocytoma cyst as is reflected in the name (PheochromoCytoma). As this cyst occurred in an adrenal gland, the resulting cell line should supposedly contain norepinephrine and epinephrine. However, determination of the transmitter species with high-pressure liquid chromatography (HPLC) and capillary electrophoresis (CE) have shown an abundance of dopamine in many sublines of PC12 cells.[94–96]

Since the initial isolation, PC12 cells have become a widely used cell model for studying exocytosis and related processes.[97,98] As it is derived from a malignant cyst, the cells divide continuously and are thereby less labour intensive to culture compared to primary cell cultures. In addition, genetic manipulation and the possibility to create cell lines with specific proteins expressed or removed further extends the scientific uses of the cells. Being cancerous could also be a potential downside, as altered metabolism and changes in protein expression might make this cell line diverge from the behaviours of

healthy cells.[99] The age of the cell line could also be a disadvantage, as potential sub-cell lines might have formed during the continued culturing, making comparison of data in the literature increasingly difficult. One example of this was demonstrated by Shoji-Kasai et al. who were able to selectively grow sub cloned PC12 cells showing vastly different responses to stimuli due to faults in calcium sensing or catecholamine metabolism.[100]

As long as these potential downsides are kept in mind, PC12 cells are a valuable model for studying the fundamental secretory processes and have been used as such for decades. One aspect that supports the further usage is the ability of PC12 cells to form processes from the cell body that resembles the growth pattern of sympathetic neurons.[93] This occurs upon treatment with nerve growth factor (NGF) and is coupled with a move of secretion from cell body to the ends of the newly formed processes.[101] The cells also express many receptors of interest in neuroscience: nicotinic, muscarinic, purinergic and dopaminergic receptors.[102–104] In addition, receptors not endogenous to PC12 cells can be expressed using genetic manipulation as mentioned earlier.[105] Although the expressed receptors can be used to stimulate the cell and initiate the exocytotic pathway, most commonly a high concentration K^+ solution or electrical stimulation are used to quickly depolarize the plasma membrane and open the voltage-gated calcium channels. As a comparison, stimulation using the nicotinic or muscarinic receptors results in a delayed exocytotic response.[23]

3.2 Chromaffin cells

Chromaffin cells are found in the medulla of the adrenal gland, a small endocrine organ placed in close proximity to the kidneys. These neuroendocrine cells contain and release primarily norepinephrine and epinephrine when the gland is stimulated by the splanchnic nerve. It should be noted that the adrenal gland also contains cortex cells that have the same morphology as chromaffin cells and these cells secret various steroid hormones (Fig. 3.1). Chromaffin cells are typically grown as primary cell cultures and as such they can be obtained from a variety of sources. Most often they come from the adrenal glands of rats, mice, and cows. Other sources, such as cat, guinea-pig and even human, have been described as well.[106–108] The resulting chromaffin cell cultures could have different expression of receptors and ion channels depending on which animal they originate from.[108,109]

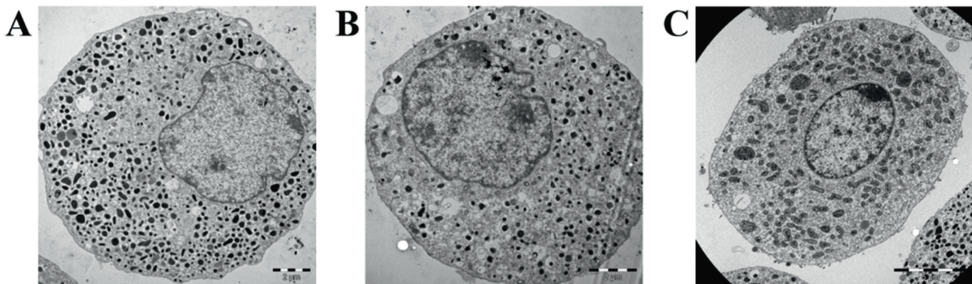

Figure 3.1 TEM micrographs of secretory cells found in the medulla and cortex of adrenal glands. A, adrenergic cell B, noradrenergic cell C, cortex cell. Scale bars are 2 µm in A and B, 5 µm in C. Reproduced with permission from ref 110.

This is an important factor to consider when interpreting data derived from chromaffin cell experiments.

The secretory machinery consists of the same proteins that were mentioned in section 2.2 and results in the release of transmitter content from LDCVs. Besides small molecules, the proteinic dense core in LDCVs can be released as well, allowing peptides such as vasostatin, catestatin and pancreastatin to act as messengers. These peptides are released into the bloodstream and affect bodily functions such as blood pressure, hormone release, glucose balance and others.[111] As with the PC12 cells, chromaffin cells can be stimulated in culture using varying methods. Most commonly electrical

activation or depolarization with a high concentration K^+ solution is used, but application of acetylcholine or Ba^{2+} also results in exocytotic release. Exactly how polyvalent metal ions initiate the process have been studied and debated for some time with evidence pointing both that Ba^{2+} acts in a competitive manner to Ca^{2+} in the exocytotic machinery or that it has a different site of action such as causing release of Ca^{2+} from intracellular calcium stores.[112–114]

The advantage of studying cellular communication in chromaffin cells is that as a primary cell line, they more closely resemble healthy cells. In addition, chromaffin cell vesicles are bigger and contain a larger amount of catecholamines making them easier to detect with analytical methods. Limitations arise from a primary cell line as well, causing the cells to have a short life span and increasing the work needed as new cultures have to be produced regularly.

3.3 *Drosophila melanogaster*

The invertebrate *Drosophila melanogaster*, fruit fly, have been used as a model organism in science for a long time. Initially it was eagerly used in genetic research as its short lifespan (~10 days from egg to adult), large amounts of progeny, and few genetic redundancies made it possible to quickly concoct and analyse genetic variations.[115] These features also contributed to the popularity of using *Drosophila* in other fields as well. Compared with rat or mice models, creating a new genotypic line of flies takes a couple of months instead of years. With regard to humans and their related diseases, *Drosophila* shares a surprisingly high biological similarity as has been showed in several studies. Reiter et al. found that 77% of the studied disease-related proteins in humans had a related sequence in *Drosophila* and Rubin et al. found protein orthologs to 117 of the 289 chosen disease related genes.[116,117] The similarity in molecular biology and the ease with which *Drosophila* are cultured and grown in the lab makes it a highly relevant research subject.

Despite all the similarities, there are also differences and limitations to using *Drosophila* as a model for human functions and diseases. *Drosophila* is an invertebrate and therefore lacks some of the more complex functions of mammals in general and humans in particular. The *Drosophila* genome has only 4 chromosomes compared with the 46 human chromosomes, illustrating the biological simplification made when studying *Drosophila*. The higher cognitive processes are also somewhat limited in *Drosophila*. Although they do exhibit some social behaviour and learning capabilities, they fall short when comparing to humans, making certain psychiatric or neurological diseases difficult to model in the fly.[118] Another striking difference is the absence of blood and a greatly dissimilar breathing apparatus in *Drosophila*. Instead of using lungs and blood with hemoglobin, fruit flies have trachea and hemolymph that carries oxygen and nutrients to organs.

The life cycle of a fruit fly is illustrated in Fig. 3.2 and starts as an embryo or egg, in which form it stays for typically 24 hours depending on the temperature; warmer climate speeds up the maturation as well as shortens the life span of the fly. When the egg hatches, a larva appears. *Drosophila* larvae go through 3 stages, or instar, each one ending with a melt. During the melt the larva has grown enough to need to change their

entire outer skin for a new larger one. The time as a larva is largely spent eating and growing, but at a point during the third instar the larva crawls out of the food to find a dry spot to pupate. This third instar is in fact the largest stage in the fly's life by volume and a common stage at which to pick subjects for research. In total, the larval stage lasts for ~4 days and when it is ready a hard-shell forms and the larva becomes a pupa. Inside the pupa, the larva undergoes metamorphosis and completely changes its biological build. This typically takes 5 days after which an adult fly emerges.[115] The cycle can then start over, with new progenies being produced over the rest of the fly's life span.

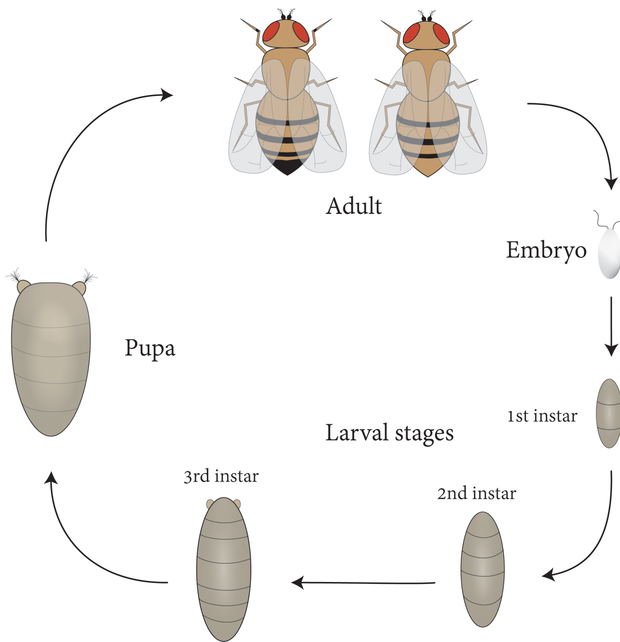

Figure 3.2 Illustration of the *Drosophila* life cycle. The time to develop from an embryo to an adult is ~10 days. Typically, the 3rd instar or the adult fly are used as model organisms in neuroscience.

Third instar larva and adult flies have been most used as models in neuroscience related research and thus a more detailed description of their nervous systems is in order. In larvae, the nervous system consists of a central nervous system of brain lobes and the ventral nerve cord as well as a peripheral nervous system through the body tissues. The larval brain has been estimated to comprise 10 000-15 000 neurons, a far cry

from the more than 100 billion neurons in a human brain.[119,120] The brain regions of the larva contain several different neurotransmitters such as octopamine, dopamine, serotonin, tyramine, glutamate, and acetylcholine.[121–123] The larvae also have nerves running from the brain to the muscles in the body wall. These form connections known as boutons or varicosities where signalling between the nerve and the muscle occurs at neuromuscular junctions (NMJ). Boutons have different functions and releasable transmitters; Type Ib and Is varicosities release glutamate, type II release glutamate/peptide/octopamine, and type III release peptides.[124–126] The muscular localization of type II boutons is illustrated in Fig. 3.3A-C.

Much like the larval brain, the adult fly brain uses glutamate, acetylcholine, dopamine, GABA, histamine, octopamine and serotonin.[123,127,128] However, the adult fly nervous system is somewhat more complex with ~100 000 neurons in the brain itself (Fig. 3.3D).[129] The adult brain is also more organized with highly specialized regions for e.g. short- and long-term memories and courtship behaviour.[130,131]

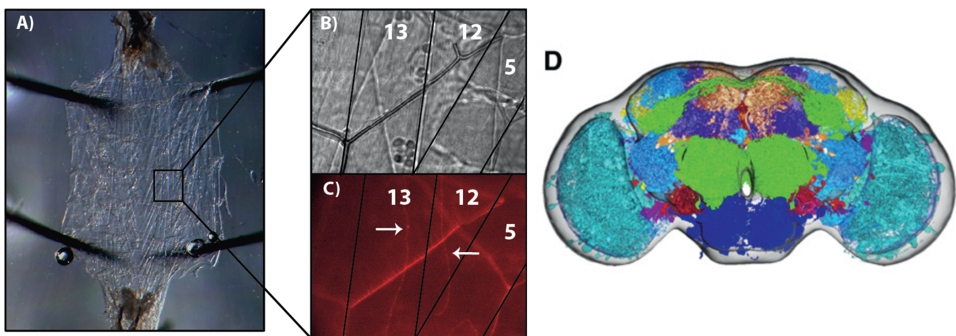

Figure 3.3 *Drosophila* nervous systems. A, the body wall of a dissected 3rd instar larva. B, Brightfield image of the larva muscles 5, 12, and 13. C, the corresponding fluorescence image to B, showing genetically labelled type II boutons (arrows). D, a combined reconstruction of the adult fly brain indicating various brain regions in different colour. For instance, green in centre: antennal lobes, turquoise: medulla, dark blue: subesophageal ganglion, purple: mushroom bodies and calyx. Reproduced with permission from ref 129.

As mentioned previously, one major benefit of using *Drosophila melanogaster* as a model system is the ease of which new genotypes can be created. Through the creation of knock-outs, mutations, and expression of exogenous proteins, this system can be

adapted for studies of a wide variety of biological processes. However, in order to have a finely tuned model, the genetic modification needs to be specific. One way of achieving this specificity is by using an expression technique called the UAS-Gal4 system. Even though this system was first discovered in yeast, it was quickly adapted for *Drosophila* usage.[132] The system is based on two parts, of which the first is the transcriptional activator Gal4. The Gal4 gene is selectively inserted into the genome to consistently express the activator Gal4 in a specific tissue or cell type. The second part is the binding site for Gal4, called the upstream activation sequence (UAS), which is a genetic sequence that Gal4 binds to and thereby activate expression of downstream genes. The UAS sequence is placed right before the gene of interest, the reporter gene, and is present throughout the organism. When both UAS and Gal4 are present in a cell, spatially specific expression of the reporter gene occurs.[132]

One example of where specific expression is necessary is in the field of optogenetics, where light-sensitive ion channels are used to depolarize and activate cells. The most common of these channels are called channelrhodopsin 1 and 2 and since their discovery a plethora of chimeras and mutants have been developed.[133–136] Through clever usage of the UAS-Gal4 system it is therefore possible to clone and produce fly lines where specific neurons are light activated and visible with fluorescence as is illustrated in Fig. 3.4. The combination of genetic modification and optogenetics turns out to be highly useful when probing the storage and release of transmitters in the *Drosophila* nervous system with electrochemistry (**paper VI**).[137]

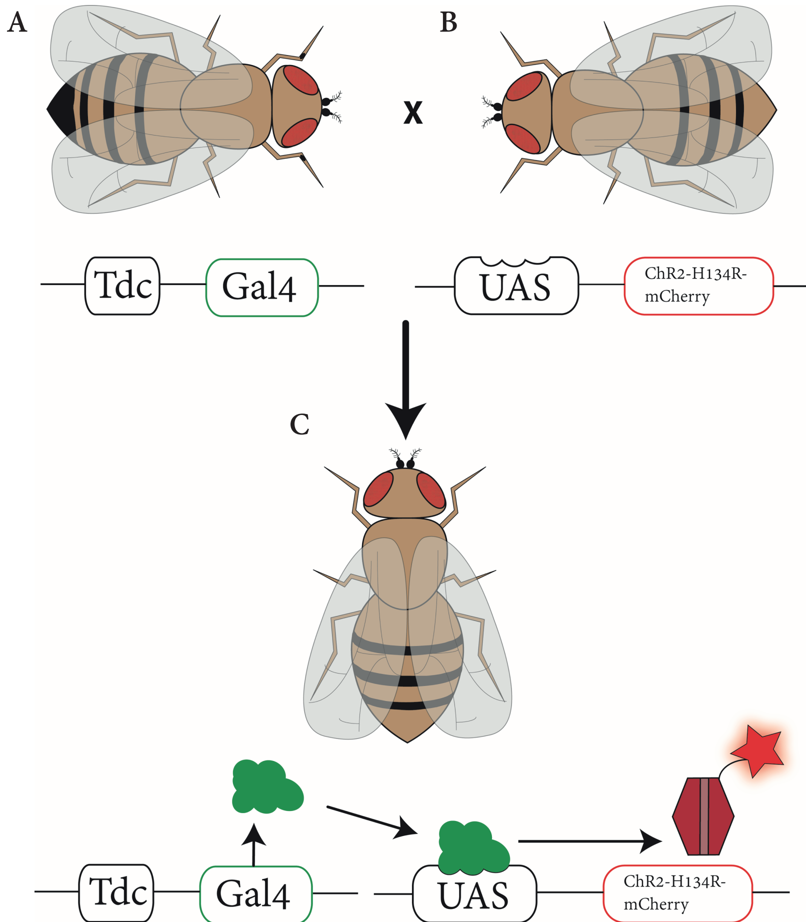

Figure 3.4 An example of optogenetics with the UAS-Gal4 system in *Drosophila*. AB, adults of genotypes for either the Gal4 and cell specific gene sequence (tyrosine decarboxylase: Tdc) or the UAS sequence and reporter gene (ChR2-H134R-mCherry) are crossed. C, In the resulting progeny the Gal4 promotor activates tissue specific transcription of the reporter gene which in this case is a fluorescently labelled ion channel.

Chapter 4. Electrochemical Analysis

4.1 Theory of electrochemical analysis

Several different biological model systems were described in chapter 3 and a number of analytical methods have been developed to use these to study cellular communication and exocytosis. The focus of this chapter is on electrochemical methods, which involve measuring the charge, potential, or current during certain conditions at an electrode surface. In addition to some fundamental concepts, the commonly used techniques of cyclic voltammetry and amperometry will be addressed. Finally, although not traditionally regarded as an electrochemical technique, the method of patch clamp is briefly described due to its widespread use regarding cellular communication.

As was mentioned previously in the thesis, there are a large number of molecules that act as transmitters or otherwise regulate the chemical signals between cells (1.3 Types of transmitters). Although not all of them are suitable for electrochemical analysis, i.e. electroactive, a considerable number of them are. An electroactive molecule has the ability to either be oxidised or reduced in a given potential window. Some of the electroactive molecules commonly studied using electrochemistry are dopamine, norepinephrine, epinephrine, serotonin, histamine, ascorbate, and the invertebrate transmitter octopamine. Besides these there are smaller molecules such as NO, O_2, and H_2O_2 that are of general biological interest as they can provide insight to the metabolism of cells or tissue.[138] Examples of redox reactions for some of the molecules above are shown in Fig. 4.1.

Besides the electroactive molecules, electrode surfaces can be modified with, for instance, enzymes or other biological molecules to measure molecules that are not electroactive in themselves. For enzyme modification, this works by catalysing a reaction that creates a product or biproduct that is electroactive and subsequently detected. Ideally the measured response is proportional to the amount of the original, non-electroactive, molecule. Use of this type of measurement further broadens the

application of electrochemical analysis to molecules such as acetylcholine, glutamate, and glucose.[139]

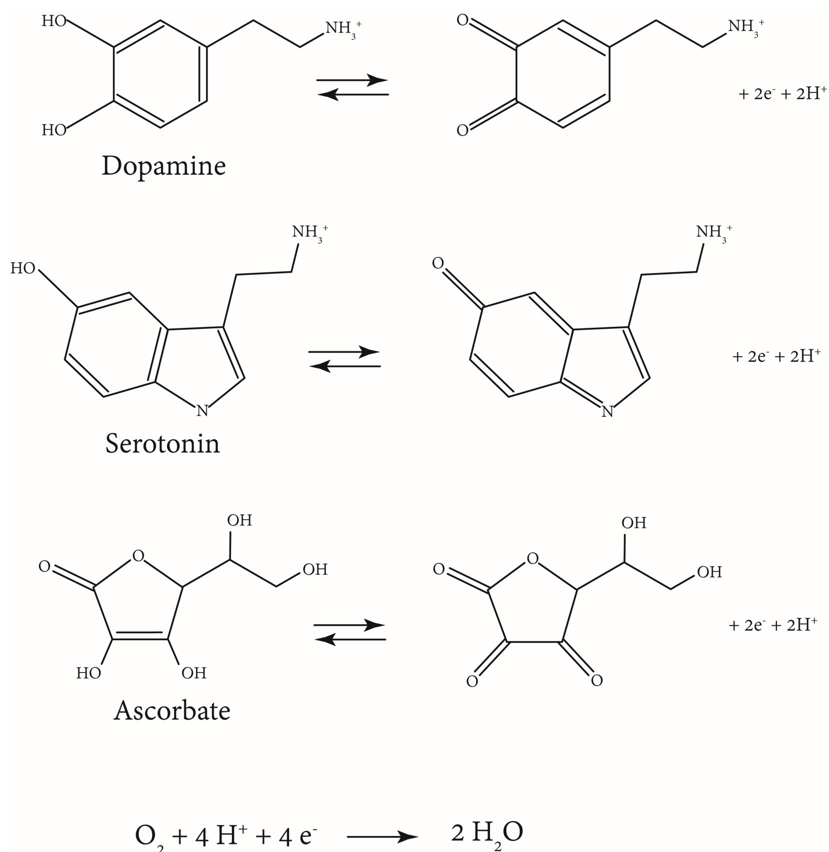

Figure 4.1 Examples of redox reactions for the biologically relevant molecules dopamine, serotonin, ascorbate, and oxygen.

4.1.1 Faradaic processes

For the following discussion of electrochemical techniques, it is of use to separate and briefly discuss Faradaic *vs* non-Faradaic processes. As is indicated by the name, Faradic processes are related to a redox reaction through Faraday's law. This law states that the observed current in an experiment is proportional to the amount of analyte undergoing redox reaction. Thus, current measured from a Faradaic process is known

as Faradaic current. Although simply stated, several complex physicochemical aspects of an experiment influence and govern the rate of the redox reaction. Some of these aspects will be described concisely below.[140,141]

One central concept in chemistry is that of thermodynamics, and electrochemistry is not an exception. Thermodynamics largely determine if a redox reaction is favourable or not. As the free energy of a reaction can be cumbersome to estimate and work with, the equation derived from free energy expressions known as the Nernst equation is commonly used for electrochemical analysis. The Nernst equation relates the formal potential and reaction quotient of a reaction to the potential of an electrochemical cell. If the reaction is in equilibrium, the reaction quotient is equal to the equilibrium potential and the potential across the electrochemical cell zero.[140] In addition to thermodynamics, the kinetics of a reaction also need consideration. Even though a reaction is thermodynamically favourable, it might not occur at any significant rate.[140,141]

As electrochemical measurements occur at an electrode surface, mass-transport of the analyte to the surface can also influence the amount of analyte available for subsequent reaction. Mass-transport of analytes in a solution occurs through three main ways. The first is convection, meaning physical stirring or movement of the solution to move analytes around the bulk solution and to the electrode surface. The second is migration, in which charged species move under the influence of an electric field. The third and last is diffusion, which is the random movement of molecules in a solution. Through the random movement of molecules across a concentration gradient, the differences in concentration will eventually equalise.[140] In an electrochemical reaction, the reaction at the electrode causes the reactant concentration to go to zero and so diffusion goes from solution to the electrode surface. Interestingly, the shape and size of the electrode affect the diffusion of analyte. Whereas the diffusion towards macroelectrode is linear, microelectrodes also have a contribution of radial diffusion that makes the mass-transport more effective (Fig. 4.2).

Depending on the specific details of an electrochemical experiment such as the redox reaction in question and the rate of mass-transport in the system, one aspect will be slower than the others and therefore rate limiting. In addition, other surface reactions

such as adsorption, desorption or electrodeposition can also affect the rate of the reaction to further complicate the task of assessing the contribution of Faradaic processes to the measured response.[140]

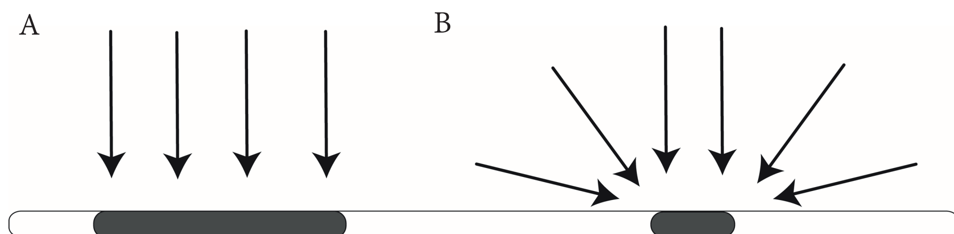

Figure 4.2 Diffusion at A, a macroelectrode where linear diffusion is predominant or B, a microelectrode where radial diffusion also contributes to the mass-transport.

4.1.2 Non-Faradaic processes

Various surface reactions, changes in the applied potential, or in the constitution of the solution can also be part of non-Faradaic processes in an electrochemical experiment. Unlike the Faradic processes, these do not involve a typical redox reaction and the subsequent transfer of electrons but can still cause significant effects on the measured result. This is especially troublesome as the contribution from faradaic and non-Faradaic processes can be difficult to untangle.[140]

The organisation of charged species at the electrode surface and nearby solution, known as the electrical double layer, is one feature contributing to the non-Faradaic response. Due to the electrostatic effects at the electrode surface when a potential is applied, charged species will organise accordingly and essentially make the electrode-solution interface act as a capacitor. In electronics, application of a potential charges the capacitor and during the build-up of charge a current flow, the charging current. Once the capacitor is charged the current diminishes.[140,141]

This phenomenon also occurs at the electrode-solution interface, but unlike an electronic capacitor, the build-up of charge and charging current is dependent on the applied potential. For an electrochemical measurement, this means that as soon as the potential is changed, the capacitive properties of the electrode-solution interface will

shift along with the occurrence of the charging current needed to re-establish the charge.[140,141] That charging current cannot be considered irrelevant is made clear when the electrochemical technique of cyclic voltammetry is discussed next.

4.2 Cyclic voltammetry

4.2.1 Methodology of cyclic voltammetry

In cyclic voltammetry (CV), the applied potential is actively scanned from one potential to another and then back (Fig. 4.3A), while the current is measured. The data are then plotted in a current *vs* potential plot as a cyclic voltammogram (Fig. 4.3B). By scanning across a potential window, information concerning the redox reaction is obtained. In fact, as many reactions differ in redox potential, CV can be used as a qualitative method to identify electroactive compounds based on the appearance of the voltammogram and position of the peaks at which reduction or oxidation occurs.[140,141] CV can also be used as a quantitative method as the measured current at a redox peak or plateau is proportional to the concentration of analyte in the solution. In addition, this current is also related to the electrode area and can as such be useful for characterising electrodes.

Cyclic voltammograms differ in appearance depending on whether or not the measurement is done using a macroelectrode or a microelectrode. The peak in Fig. 4.3.B is caused by the limitation of mass-transport of the analyte. With an increasing potential, the reaction is further driven in one direction and there is a depletion of analyte at the electrode surface. If the mass-transport in the system is not fast enough, this depletion causes a decrease in measured current. As mentioned previously, (4.1.1 Faradaic processes), diffusion at microelectrodes is more effective due to the contribution of radial diffusion. Therefore, in CV measurements using microelectrodes distinct redox peaks are not observed and the voltammogram forms a sigmoidal shape instead (Fig. 4.3.C).

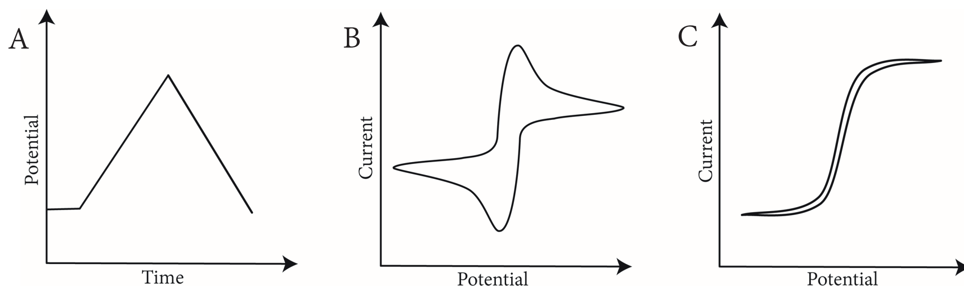

Figure 4.3 A, Scheme of a triangular potential waveform used in CV. B, Resulting voltammogram from a macroelectrode. C, Sigmoidal voltammogram from a microelectrode.

Several features can also be altered in CV to optimise the method for a specific purpose. For biological uses this can be highly interesting as the samples can be complex in nature, containing several electroactive molecules that all have their own electroactive properties. One of feature to optimise is the waveform of the applied potential. For instance, development of more selective or sensitive waveforms for detection of dopamine, serotonin, histamine, and octopamine have been reported.[138,142–145]

4.2.2 Advantages and drawbacks of CV

Perhaps the main advantage of CV is that is can give both qualitative and quantitative information about electroactive molecules that are present. However, not a single technique is without both advantages and drawbacks and here some of them are discussed.

The speed with which the potential is scanned, scan rate, can be used as an optimisation parameter in CV. One obvious effect of increasing the scan rate is an increase in temporal resolution, fast scan cyclic voltammetry (FSCV). Many *in vivo* studies from recent years use FSCV to obtain approximately 10 voltammograms per second.[138,146,147] Although altering the scan rate can be useful, it should also be noted that rapid changes in potential can cause interference in the measurement. This is due to the capacitive properties of the electrode-solution interface as discussed previously (4.1.2 Non-Faradaic processes) resulting in a charging current that can be large enough to obscure the signal from the analyte.[140] One solution to this issue is to use background subtraction to estimate and delete the contribution of charging current to the measurement, although this requires some additional considerations when designing an experiment.[148]

A final advantage to the use of CV is its ability to measure with great detail chemical reactions following the redox reactions of biologically relevant molecules. Some molecules undergo additional conformational changes or reactions after a redox reaction and these can be studied with CV.[140,141] One example of this is elucidating the oxidation mechanism of octopamine which forms radicals that subsequently dimerise and can foul an electrode by polymerisation.[45]

4.2.3 Applications of CV

Besides its widespread use to characterise the electrochemical properties of molecules and electrodes alike, the method of CV has grown increasingly important for studying cellular communication, for instance in the brains of model organisms. By implanting an electrode in a specific region of a brain, several aspects such as spontaneous transmitter transients and pharmacological effects on this release can be studied.[149,150] Additionally, behavioural studies of awake and moving animals can be performed. By exposing the organism to a positive or negative cue while at the same time measuring changes in the levels of e.g. dopamine, the intricate reward system of the brain has been studied.[146,147]

Although transmitter release is important, resetting of the signalling pathways by removing extracellular transmitters is equally important. The question of transmitter uptake has also been addressed with CV. By measuring the disappearance of the dopamine signal, pharmacological effects on transmitter uptake can be quantified.[151] This has been performed, for example, in the brains of adult *Drosophila* (fruit flies). Even though a significantly smaller organism than rats or mice, the same procedure of surgically inserting a microelectrode into a dopaminergic region has allowed determination of dopamine uptake in both genetically modified flies with an impaired dopamine transporter and after pharmacological treatment.[152–154]

The selectivity of CV and the possibility to study differences in electrochemical reactions is an important issue for *in vivo* measurements. In for instance the brain, ascorbate is a common interferent as it is present at quite high concentrations and also oxidise at a similar potential as dopamine. Ascorbate and dopamine, however, differ in the rate of their oxidation, so by simply increasing the scan rate of a CV measurement decreases the interference and provide better dopamine selectivity.[138] Another example used the rate differences in cyclisation for the highly similar molecules norepinephrine and epinephrine to distinguish and detect both these in chromaffin cell cultures.[155]

4.3 Amperometry

4.3.1 Methodology of amperometry

Another electrochemical method used for studying cellular communication is amperometry. In contrast to cyclic voltammetry, the potential is in this case kept constant while the current is measured. Any electroactive molecules present will react causing a change in current.[138,140]

Single cell amperometry (SCA) was first described in 1990 and consists of placing an electrode on top of a secretory cell. A potential is applied to the electrode and the cell is stimulated to initiate exocytosis. Commonly an overpotential is applied to facilitate rapid redox reactions. As the vesicles release transmitters, these diffuse to the surface of the electrode to react (most often oxidise) and the resulting current is detected.[12] As the electrode typically is placed in close proximity of the plasma membrane, the set up can be regarded as a simplified synapse reconstruction.

High enough sampling rates allow individual exocytotic events to be observed and are sometimes denoted as peaks in an amperometric current-time trace. These peaks can be further analysed to provide detailed information concerning the exocytotic process. For instance, integrating the area under the peak gives the total charge of the event. If the redox reaction is known, the charge can then be related to the number of molecules detected through Faraday's law. Details such as the rise time (t_{rise}), fall time (t_{fall}), width($t_{1/2}$), and maximum current (I_{max}) can also be extracted from the peak for further analysis (Fig. 4.4).[156]

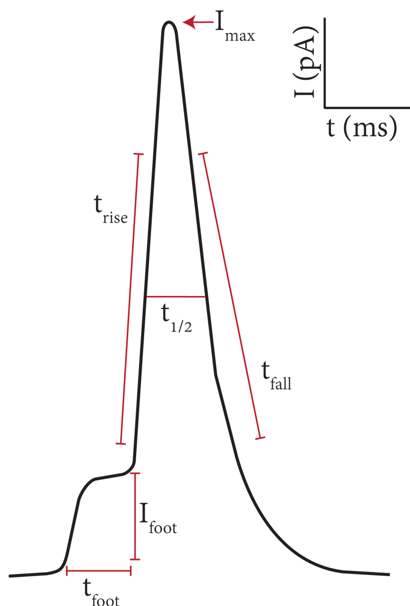

Figure 4.4 Illustration of an amperometric peak as measured in SCA with some relevant features highlighted.

As the current is related to the number of transmitters reaching the electrode, the amperometric peaks are also a representation of the dynamic processes of the fusion pore and the escape of transmitters from the vesicle. As seen in Fig 4.5, during the initial formation, the fusion pore is small with only a small flux of transmitters. This small increment before the peak is known as a foot and can be further analysed as to the duration (t_{foot}), maximum current (I_{foot}), and charge involved. As the fusion pore expands, the number of released transmitters increases resulting in an increase of the current. Finally, most often is appears that the combination of closure of the fusion pore and the diffusion of transmitters cause the current to slowly decrease in a decaying manner.[157–159] In a small percentage of events the pore opens all the way leading to complete release. This was discussed in the section on exocytosis.

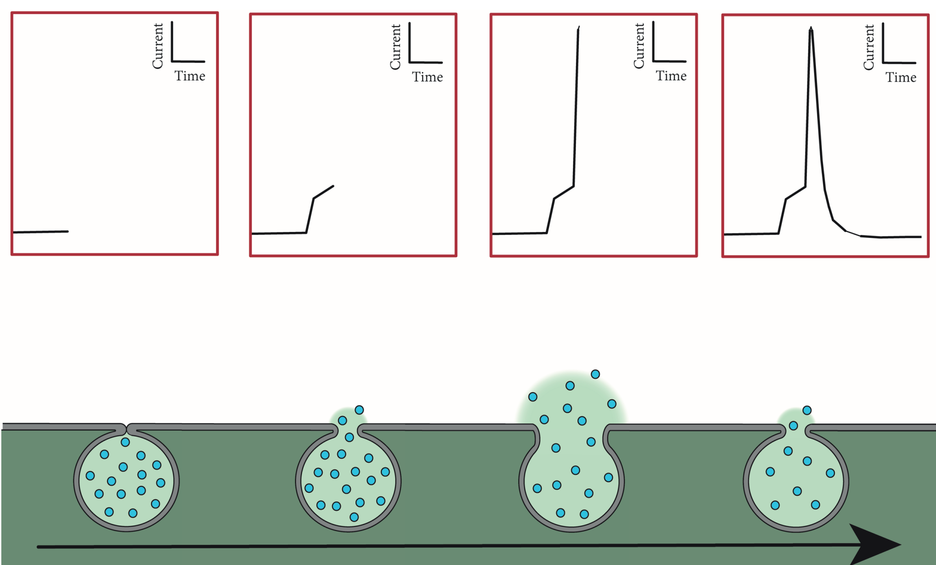

Figure 4.5 Illustration of the correlation of an amperometric peak (top row) and exocytotic release (bottom row).

4.3.2 Advantages and drawbacks of amperometry

One major advantage of amperometry is the high temporal resolution. For typical experiments in a lab the current can be measured at 10 or even 20 kHz, compared to 10

Hz for standard CV measurement, allowing enough data points per exocytotic event to pick out the very subtle and fast changes in transmitter flux.[157] This high temporal resolution is, for instance, required for separating the different modes of exocytosis discussed in 2.3 Regulation of exocytosis. A fusion pore that is open for a short period of time and releases a small amount of transmitter molecules will result in a smaller and faster peak than for a pore that is open for longer.[160]

Amperometry is also quantitative enough to make it possible to calculate the number of molecules that are released during exocytosis. However, the lack of qualitative ability is a disadvantage. The application of a constant potential over time does not provide information about what electroactive molecule is actually detected.[138] In fact, if several transmitters are present at the same time and have similar redox potentials, amperometry cannot distinguish them from each other. Pharmacology can be used, to some extent, to provide insight as to which molecule is present by selectively blocking or enhancing the presence of a specific molecule.[137]

4.3.3 Applications of amperometry

Since the initial application of SCA at chromaffin cells, this technique has been further applied to use on a wide variety of other secretory model systems such as PC12 cells, *Drosophila* larva NMJ, neuronal synapses, pancreatic β-cells, and mast cells.[97,137,161–163] These types of SCA studies allow studies of both biological, chemical and physical factors that influence the exocytotic process, such as genetical modification, receptor influence, metabolic changes, protein interactions, pharmacological treatments, and membrane fluidity (**papers I, II, III, IV, V, VI**).[57,74,88,91,164,165]

Amperometry has also been used as a method to determine the total content of transmitters in vesicles. It was observed that lysing cells and separating the vesicles followed by electrochemical detection using amperometry made it possible to measure the vesicular transmitter content and compare it to how much was released during exocytosis.[166] Later it was realised that the separation of vesicles was not required, and by simply isolating vesicles from cells and placing a polarised electrode in the suspension resulted in peaks corresponding to the rupture of individual vesicles in a technique called vesicle impact electrochemical cytometry (VIEC).[84] This realisation also led to

further *in situ* measurements called intracellular vesicle impact electrochemical cytometry (IVIEC) using a needle-shaped, sharp electrode to pierce the plasma membrane of a cell in order to measure the vesicle content. By measuring vesicular content inside the cell the possibility of transmitter leakage out of the vesicle during isolation is minimised and the method is easily combined with SCA.[85]

By combining SCA with VIEC and/or IVIEC techniques, a comparison to determine the fraction or percentage of released transmitters can be done. This is highly valuable as it makes it possible to quantify partial release of exocytosis. Although determining the partiality of single vesicles is still in development, an average release percentage from a cell or cell population can be obtained this way.[85] Besides the chromaffin and PC12 cell models, the combination of SCA with IVIEC has been applied to *Drosophila* larva NMJ. By using a genetically modified fly larvae in which the NMJ is fluorescently labelled, a sharp electrode was used to pierce the NMJ and access the vesicles inside. Comparison between the transmitter measured in the vesicles and previously published data on the amount of release from the NMJ revealed that only a minute fraction of the vesicular content is released in this model system (**paper VI**).

In addition, effects observed after pharmacological manipulation or other treatments can be further pinpointed by determining if the cause is due to differences in vesicular storage or in the release mechanism itself. By also treating isolated vesicles and performing VIEC, the effects caused by the cellular machinery are taken away enabling one to estimate how the vesicular level is altered (**papers III, IV**).[164,167,168]

4.4 Patch clamp

4.4.1 Methodology of patch clamp

Although not a typical electrochemical technique, a brief description of patch clamp is relevant in a thesis focusing on cellular communication. This is due to the long and extensive use of patch clamp to study various features and phenomena related to cellular communication, including exocytosis.

Unlike the previous methods of CV and amperometry, patch clamp uses variations of Ohm's law to study effects occurring at or across the plasma membrane. Another difference is the use of a narrow glass pipette instead of an electrode to perform the measurements. This pipette is brought in close proximity to the cell or tissue under investigation and depending on the type of scientific question in mind, different configurations of patch clamp can be achieved. The pipette can either be brought close enough to the membrane to form a resistive seal, called cell-attached, or can break through the membrane completely so the solution in the pipette is in contact with the cytosol, called whole-cell configuration. In addition, patches of membrane can be excised from the cell and studied without the rest of the cell.[169]

As the pipette forms a seal with the plasma membrane, an electrical circuit in which either the potential or the current flow can be controlled. Depending on the setup of the experiment, the current flowing through membrane channels, the voltage across the plasma membrane, or the capacitance of the membrane can be determined.[169]

4.4.2 Advantages and drawbacks of patch clamp

The advantages of patch clamp are to some extent complementary to those of the previously mentioned techniques. As mentioned in 1.2 Chemical signals in biological systems, cellular communication is strongly linked to the potential across the plasma membrane and patch clamp can be used to measure not only the potential but also the ion fluxes across the channels that uphold this potential. In addition, patch clamp can be set up to measure changes due to endocytosis as well as exocytosis in order to study a larger span of the vesicle life cycle.[169,170]

Subcellular spatial resolution can also be obtained by excising patches of plasma membrane. Although patch clamp also can measure the changes involved in exocytosis

of non-electroactive species, the method is not selective or specific enough to identify the transmitter involved nor does it provide quantitative chemical information in the same way as CV or amperometry.[138,169]

4.4.3 Applications of patch clamp

Some applications of patch clamp for studying cellular communication have already been mentioned. Measuring ion fluxes such as those of calcium can be highly interesting due to the implied role of calcium as the trigger for initiation of exocytosis.[171] As the fusion pore can be thought of a small channel with a flux of molecules, patch clamp can also be used to indirectly study the efflux of transmitters and pore dynamics as conductance during exocytosis.[172]

Changes in measured capacitance related to exo- or endocytosis are also of interest as capacitance is related to the area of the membrane therefore making it possible to estimate the sizes of the vesicles involved in these processes. However, it should be noted that due to the signal-to-noise ratio of patch clamp measurements, smaller vesicles might not be distinguishable.[138]

Patch clamp has also been used in combination with amperometry in what is called patch amperometry. This method consists of positioning a carbon-fiber microelectrode inside the patch pipette and then forming a seal with the membrane in the cell-attached configuration. As vesicles under the pipette fuse with the plasma membrane, the increased capacitance steps can be related to amperometric spikes from released transmitters into the pipette as these are detected by the electrode.[65]

The possibility to measure ion fluxes as well as quantitatively and qualitatively study transmitter release and uptake in biological model systems have made the electrochemical techniques discussed in this chapter highly valuable in this field. Although they complement each other to some extent, scientific questions as complex as that of cellular communication benefit from using a variety of analytical techniques. Some of these techniques focusing on imaging biological samples will be covered in the next chapter.

Chapter 5. Imaging Techniques

While electrochemical techniques can provide much insight into the quantities and temporal resolution of the release of transmitters in secretory cells, spatial resolution is often lacking or limited to single cells. In addition, the cellular distribution of proteins and other potential regulatory factors is highly interesting to understand the fundamental processes governing exocytosis and cellular communication. Several cellular areas such as the synapse or active zones (1.1 Cell-to-cell communication) are in fact highly spatially organised and the transport of proteins and organelles to the proper sites of action is vital for proper cellular function. This chapter therefore aims to provide an overview of common imaging techniques and practices used in the field. Fluorescence microscopy, electron microscopy, and imaging mass spectrometry are addressed and illustrated with relevant applications and recent developments.

5.1 Fluorescence microscopy

5.1.1 Theory of fluorescence microscopy

Imaging using fluorescence microscopy has been a popular method to use for addressing problems in life science for a long time now. As such it has also been applied to study exocytosis and intracellular signalling. Fluorescence is induced by an electronic transition of a molecule from an electronic ground state to an excited state after which the excess energy is released back as a photon as the molecule transitions back to its ground state. Typically, some of the initially absorbed energy is lost through vibrational processes and heat dissipation, causing the emitted photon to have less energy and thereby be of longer wavelength.[173,174] This emitted light can then be measured using a detector (Fig. 5.1).

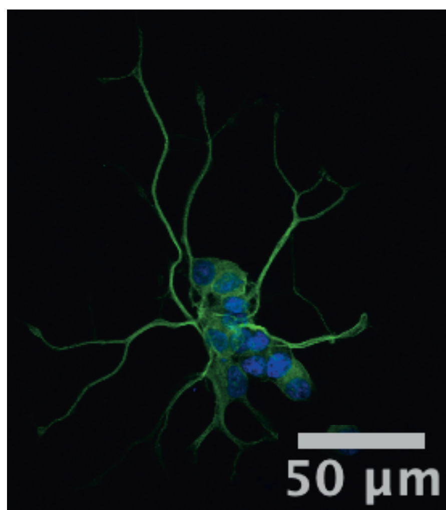

Figure 5.1 An example of fluorescence imaging from a confocal microscope. Differentiated PC12 cells have been stained to show the nucleus in blue and the cytoskeletal protein β-tubulin in green. Upon differentiation, neurite-like structures grow from the cell bodies. Image obtained at the Centre for Cellular Imaging at the University of Gothenburg.

As not all molecules have suitable fluorescent properties (see 5.1.2 Fluorescent dyes), this method allows for a fairly selective way of determining spatial and sometimes also temporal distribution of a molecule in a biological sample. In addition, as the intensity of the emitted light is proportional to the concentration of the molecule, quantitative information can be gained as well. One of the limitations, perhaps especially in relation to the field of cellular communication, is the so-called diffraction limit. This physical law, typically expressed through Abbe's law of diffraction, limits conventional fluorescence microscopy to see objects of ~200 nm in size due to the limit of diffraction which is related to the wavelength of visible light.[173]

As vesicle sizes, for instance, range 40-200 nm in diameter, the diffraction limit severely limits how detailed information can be obtained about the exocytotic process. The past couple of decades have seen some technological advances to overcome this limitation, extending the boundaries of fluorescence microscopy in biological research (see 5.1.3 Applications for studying cellular signalling and exocytosis).[173]

5.1.2 Fluorescent dyes

Dyes and fluorophores used in fluorescence microscopy can vary greatly in appearance and physicochemical properties. Depending on the aim of research, different dyes have varying advantages and disadvantages and will be described briefly below.

Small organic dyes typically make use of aromatic rings or conjugated double bonds to gain fluorescent properties.[174] These molecules can be made fairly small and their specific properties depend on the chemical structure.[174] The need for the organic dyes to be loaded into the cell and/or subcellular compartments can be a potential downside as the dye has to be able to reach the place of interest without damaging the sample. However, if a specific cellular target is considered, it is possible to design and synthesise a fluorescent molecule for that purpose. An example of this is the class of molecules called false fluorescent neurotransmitters (FFNs) (Fig 5.2A). These have similar structures to different transmitters and as such can be loaded into vesicles through the cell's endogenous transporters in a highly selective manner for subsequent imaging.[175]

Another possibility to evade the issue of loading a dye into the cell is the use of fluorescent proteins. These were initially discovered in the 60s in the jellyfish species *Aequorea* and have since then become a staple dye for use in life science.[176] Although the first protein discovered was green fluorescent protein (GFP) (Fig 5.2B), a plethora of variations have now been either discovered or developed with excitation and emission spectra ranging throughout the visible light range.[177] As a protein, these dyes can be genetically encoded to be selectively expressed in tissue or cells without further sample processing.[178] One particular benefit is the ability to combine a fluorescent protein with a target protein, effectively allowing highly specific tracking of that particular protein. One such example is the use of GFP fused to proteins related to the vesicles, chromogranin, and neuropeptide Y. This makes it possible to observe the vesicles distribution in the cell as well as to track their movements and responses to stimuli.[179] The downside of using proteins such as dyes is that they can be quite large (GFP is a > 20 kDa protein). This could be troublesome if the sheer size of the protein causes

hinderance to cellular processes and protein function or if it forms aggregates due to misfolding.[180]

Another highly specific class of dyes is based on antibodies and variations thereof. These immunologically derived proteins have an extremely high specificity for a target molecule and can be used either alone or in a two-antibody system. In contrast to the fluorescent proteins described earlier, antibodies are not genetically encoded in the sample and therefore must be loaded into the cell as with the organic dyes. As an additional downside, the size of typical antibodies (~150 kDa for an IgG antibody) typically requires cells to be fixed and permeabilized before any staining, thus making live cell imaging cumbersome.[181,182]

Ratiometric dyes can be of varying origin but are described here due to their different way of being analysed. Instead of using one wavelength to excite the fluorophore and then measuring the subsequent emission intensity at another wavelength, ratiometric dyes use a shift in the emission or excitation spectra. The shift is due to interactions with the target molecule and the magnitude of the shift is related to the amount of target molecule present in the sample. The benefit of using ratiometric dyes is that they impart less variance due to for instance sample thickness and loading efficiencies. One ratiometric dye that is often used for studying cellular signalling is Fura-2, an organic molecule used for detecting free calcium levels. In the presence of calcium ions, the excitation maxima in the spectra of Fura-2 shifts in a concentration dependent manner from 380 nm to 340 nm.[183] By successively imaging at these wavelengths, a ratio that corresponds to the level of calcium can be obtained. Due to the vital role of calcium in exocytosis, Fura-2 is a highly useful dye in this field (**papers II, III, and V**).

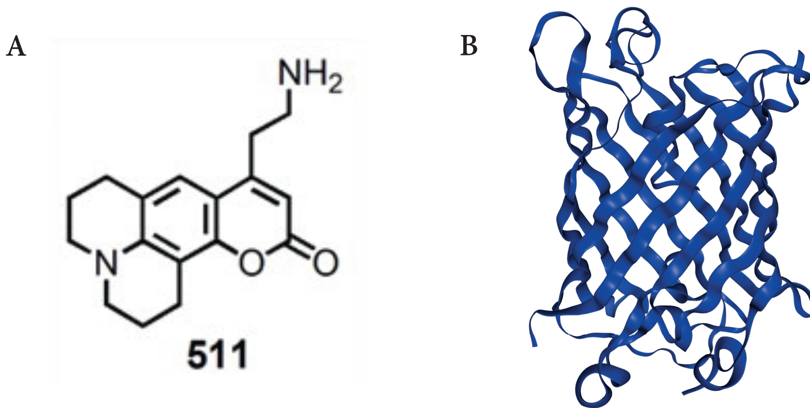

Figure 5.2 A selection of fluorescent dyes used in life science. A, the organic molecule FFN511. Reproduced with permission from ref 175. B, The crystal structure of Green Fluorescent Protein (GPF), drawn from the Protein Data Bank (rcsb.org) ID 1EMA.[184]

5.1.3 Applications for studying cellular signalling and exocytosis

In addition to a range of dyes available, there are also a number of variations in the methodologies that can be applied depending on the aim of the study and the type of sample. A number of different light sources are used as well: arc lamps, LEDs and lasers. Here some of the most common methodologies as well as some recent advances will be described in relation to their use in studying cellular signalling.

Epifluorescence microscopy is the most common method available perhaps due to its quite simple and cheap instrumentation. Typically, these microscopes are of the inverted type with the objective below the sample. The objective is used to focus the excitation light on the sample and also the emitted light back toward the detector. A dichroic mirror is placed in the beam paths leading to and from the objective to allow the excitation beam to strike the sample while keeping it from reaching the detector, thus facilitating a high signal-to noise level.[173] Confocal microscopy is perhaps the second most common method. As with epifluorescence, the instrumentation is not extravagantly expensive and is easy to use. The working principle is also quite similar to epifluorescence except lasers are often used as the light source and only a small volume of the sample is illuminated at a time. The key to this approach is that the emitted light is passed through a pinhole before reaching the detector. This feature leads to mainly

light from a tight focal point to pass the pinhole and removes most of the out of focus light, thus enhancing the contrast of the image.[173] Due to the small illuminated volume, confocal microscopy makes it possible to optically section samples to create a three-dimensional representation of the sample. This property along with the ability to image live tissue was utilized to follow vesicle maturation and fusion in a secretory *Drosophila* organ. The roles of several proteins could be elucidated based on this experimental procedure.[185]

Another way to minimize out of focus light is by using total internal reflection fluorescence microscopy (TIRFM). In this microscopic approach, the incident light beam is completely reflected by either a prism or by the angle of the light inside the objective. Thus, no direct light reaches the sample. However, as the light is reflected an electromagnetic field of the same frequency as the light known as the evanescent wave is produced.[173] As this field only penetrates ~100-200 nm into the sample, fluorophores in a small optical section of the sample close to the surface are excited. This minimizes out of focus light and increases the signal-to-noise ratio, while at the same time minimizing phototoxic effects to living cells. The obvious downside being that only a small section at the very bottom of the sample can be analysed. TIRFM has for instance been used to study vesicle movement close to the plasma membrane and the subsequent exocytotic fusion of these vesicles.[68]

While the techniques mentioned above have contributed immensely to several fields including that of cellular communication and exocytosis, they are all constrained in resolution by the previously mentioned diffraction limit. Fortunately, strategies to overcome the diffraction limit started to be developed some decades years ago. Clever ways of detecting emitted light, illuminating the sample or post processing of images all open the possibility to circumvent the diffraction limit and reach a lateral resolution of ~20 nm.[186–188] It should be noted that not all techniques are capable of providing super resolution in all dimensions and trade-offs are often necessary depending on in what direction high resolution is needed.[173] Although a detailed description of all super resolution techniques is outside the scope of this thesis, examples of super resolution techniques have been applied to cellular communication include visualization of synaptotagmin distribution after exocytosis and localisation of the dopamine

transporter in plasma membrane clusters.[189,190] With increasing technical refinements and more user-friendly and commercial instruments, super resolution techniques provide increasing amounts of information and knowledge into cellular processes. Exactly how much molecular detail into exocytosis these super resolution techniques can provide will surely become clear in the years to come.

5.2 Electron microscopy

5.2.1 Theory of electron microscopy

An alternative imaging technique is that of electron microscopy (EM). As opposed to conventional light microscopy, in EM the sample is illuminated by fast travelling electrons instead of photons. The benefit of using an electron beam is that the diffraction limit (discussed previously in section 5.1.1. Theory of fluorescence microscopy) is smaller. The limit is based on the beam wavelength and shorter wavelengths for the electron beam lead to a smaller diffraction limit and therefore higher possible spatial resolution. Due to the dual particle-wave nature of electrons as expressed by de Broglie's hypothesis, electrons can be accelerated to high speeds giving them a wavelength around 2-4 picometres depending on the power of the electron gun.[191] Based on the diffraction limit, the theoretical resolution of EM is therefore substantially improved compared to conventional light microscopy (wavelength ~400-700 nm). In relation to life science, this allows imaging of not only subcellular structures but even smaller particles and molecules such as viruses and proteins.[192,193]

There are different types of EM based on the type of electron-matter interactions that are detected. For the purpose of studying cellular communication and exocytosis, the mode of transmission electron microscopy (TEM) is often used. The principle of TEM is to use an electron beam to transmit through the sample. Areas containing elements with high atomic number scatter the incident electrons away from the detector, thus creating a darker area in the image. Lighter areas where the sample consists of elements of a low atomic number scatter less and appear translucent as most of the incident electrons reach the detector.[194] Lenses are used to shape and focus the beam and a phosphorescent screen or electron detector is used to display the sample.

Besides the drawback of not being able to do live-cell imaging with EM, two other challenges arise when applying this method to biological samples. One challenge in TEM is that electrons have a short span of interaction, meaning samples need to be thin (<100 nm is common) in order to achieve good resolution. Another challenge is that electron dense elements are not typically present in biological samples, and as such provide poor contrast in TEM.[194] To circumvent these issues, several procedures for preparation of biological samples have been developed over the years. One of the earliest

and perhaps easiest preparation methods to stabilize biological samples is to chemically fix using crosslinking agents such as glutaraldehyde or paraformaldehyde. These aldehydes form covalent bonds with primary amine groups that exist in, for instance, proteins in the sample, effectively holding the cellular structures together for the following preparation steps (Fig. 5.3).[194,195]

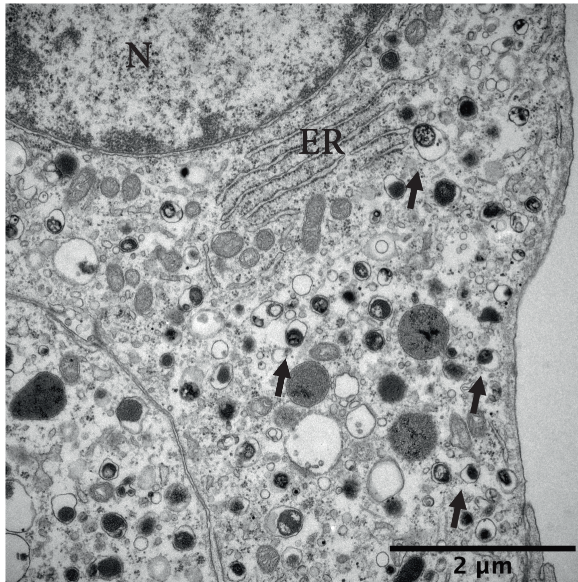

Figure 5.3 An example of a chemically fixed section of a chromaffin cell obtained with TEM. Heavy metal staining was used to provide contrast to the cellular structures and some are indicated in the figure (nucleus = N, endoplasmic reticulum = ER, large dense core vesicles = arrows). Image obtained by the EM facility EMil at Karolinska Universitetssjukhuset, Huddinge.

Other ways to stabilise samples use varying cryo-preservation techniques such as plunge freezing in liquid ethane or propane.[195] By freezing in media with properties that allow for fast heat dissipation from the sample, the water in the sample does not have time to form crystals that would adversely affect resolution and the sample is kept in a state close to its native hydrated one surrounded by vitreous ice.[196,197] This technique works best for thin samples, whereas thicker samples such as pieces of tissue or even larger cells require high pressure along with rapid freezing to hinder ice crystals from forming.[195,196] For samples that are too thick for TEM imaging, sectioning is required

after stabilization. This is typically done after the sample has been embedded in a resin.[195]

In some TEM imaging an issue of poor contrast between similar biological structures exists. To remedy this, samples can be selectively stained using heavy metal stains which scatter electrons well. For example, variations of osmium, lead, and uranium are commonly used. These typically stain different molecules and cellular structures with variable selectivity and efficiency.[195] For cryo-preserved samples that are analysed without staining, phase contrast is commonly used. This works by focusing slightly above or below the sample to shift what is known as the phase-contrast function. The effect of this shift in function, which essentially works as a band pass filter, is an emphasis or decrease of certain structures in the image obtained.[196,198]

5.2.2 Electron tomography

Although the EM procedure described above can create high resolution 2D images of biological samples, another mode, called electron tomography (ET), can be used to observe biological samples at high resolution in 3D. As cellular structures including vesicles and active zones are functionally organized with specialized areas, this information can provide valuable insight to the function and dysfunction of cellular communication.

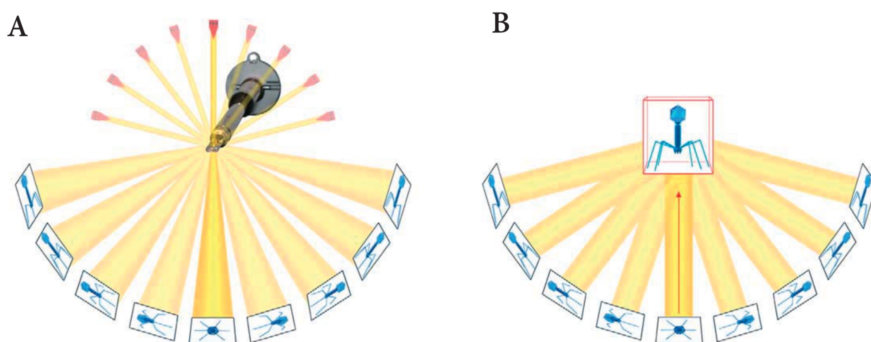

Figure 5.4 The principle of electron tomography. A, several projection images are obtained from different angles of the sample. B, Fourier transform makes it possible to back-track and combine all the projections into a 3D representation of the sample. Reproduced with permission from ref 199.

Sample preparation for ET is similar to that for TEM, but instead of obtaining one image of the sample along the vertical axis, several images are obtained each after slightly tilting the sample at different angles or image tilts (Fig. 5.4A). If enough of the volume is covered in images, calculations can be done to reconstruct the original sample volume through Fourier transform and image reconstruction (Fig. 5.4B).

Due to the sensitivity of biological samples to damage from the electron beam, electron exposure needs to be set up to minimize deterioration of the sample. During acquisition of a single EM image, the exposure of electrons can be quite high and yet still be below the recommended electron dose, resulting in images of good signal-to-noise ratios. However, in the case of ET, where the total electron dose involves an accumulation of a larger number of images, the exposure time for each image has to be significantly lower to stay below the recommended electron dose and, thus, the signal-to-noise ratio for each individual image is diminished. This also limits the number of tilt images that can actually be obtained without damaging the sample or inducing noise.[200]

Another difference in ET sample preparation is the addition of fiducial markers. These markers are needed in the reconstruction of the final tomogram as proper alignment of consecutive tilts is crucial for high quality data.[201] ET also differ in the in the instrumental requirements of high power electron guns. As the sample is tilted, the effective sample thickness (distance through the sample that the beam travels) increases, resulting in a loss of electrons that reach the detector. Thus, compared to conventional TEM a higher energy electron beam is used, or only very thin samples can be analysed.

5.2.3 Applications for studying cellular signalling and exocytosis

Due to the high resolution and potential to see small subcellular organelles using a variety of sample preparations and methodologies, the application of EM to the field of cellular communication is no surprise. One relevant application for this thesis is the use of EM to image the fusion pore as it occurs during exocytosis in several types of cells.[202–204] This type of analysis provides information about both the structure of the fusion pore as well as the region where exocytosis occurs when done in tissue sections. In addition, EM has been used to show membrane interactions and how the vesicles are brought into proximity by a protein related to exocytosis, synaptotagmin.[205]

The ultrastructure of vesicles in active zones and synapses has also been studied in several different species and types of sample preparations.[200] Here, the number or vesicles related to an active zone can be determined, as well as estimation of how many vesicles are actually close enough to the membrane to be considered docked and ready for exocytosis (see 2.2. The exocytotic pathway), and if they are physically connected to each other is also possible.[200]

One interesting development in biological imaging is to combine EM with light microscopy, correlated light and electron microscopy (CLEM). This combination of techniques takes advantage of the extensive labelling strategies available for fluorescence microscopy and the superior resolution of EM. Typically, the sample is first processed and analysed using fluorescence then fixed and analysed using EM. Alternatively, the sample is fluorescently labelled, fixed and then analysed using the both techniques. Depending on the scientific question, the fluorescent and EM techniques used can be varied to best suit the purpose of the study.[206] CLEM has been applied to several topics related to cellular communication, such as the determination of the structure of synapses and the maturation of vesicles, and since this is an area where much is unknown, it will likely continue to be developed in the future.[207,208]

5.3 Mass spectrometry

5.3.1 Theory of mass spectrometry

Mass spectrometry (MS) as an analytical technique has been around since 1912, when J.J Thomson developed the first mass spectrometer.[209] As with fluorescence microscopy, instrumental and computational advances have pushed MS further and further into fields related to life science. The basic idea of MS is to ionize molecules in the sample followed by analysis based on the mass to charge ratio (m/z) (Fig. 5.5). This makes it possible to gain a chemical map of the molecules present in the sample and, as will be described later, the method can be adapted to also provide spatial information.

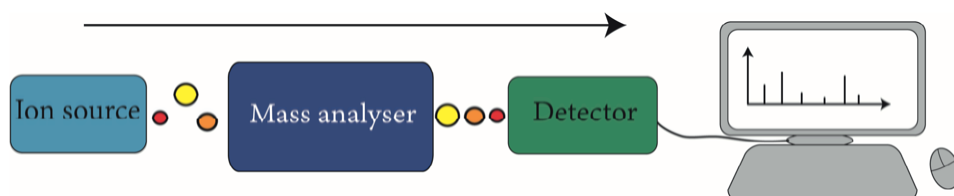

Figure 5.5 Basic schematic of a mass spectrometer. The sample is ionised and the charged species are separated in a mass analyser. After detection the mass spectrum showing intensities of the mass to charge ratios can be further interpreted.

There are a number of ways to ionise, analyse, and detect molecules, and an extensive explanation of all of these is outside the scope of this thesis. Depending on the analyte and scientific question, different ionisation approaches and mass analysers are better suited than others. For example, as larger molecules tend to break apart into fragments when ionised, different ionisation approaches are often used for small versus large species.[209] Fragmentation can be useful when trying to identify a molecular species in a fairly simple mass spectrum, but for biological samples that are generally quite complex, fragmentation can make spectra difficult to interpret.[209] Methods for sample ionisation are therefore typically divided into those that induce extensive fragmentation, hard, and those that causes less fragmentation, soft.

Matrix assisted laser desorption ionisation (MALDI) is one example of MS using soft ionisation. The desorption and ionisation of analytes is facilitated and

mediated by the addition of a matrix which has an absorption spectrum that corresponds to the incident light of the laser used.[210,211] Depending on the matrix used, different types of analytes can be preferentially detected. One advantage of MALDI is the ability to detect high mass analytes such as proteins which would most likely fragment using harder ionisation methods.[212,213] The downside of MALDI is that typical spatial resolution is around 20-50 μm, making subcellular or even cellular distributions difficult to distinguish. This limitation is in part due to the size of the laser beam used and the crystals formed by the matrix as well as the diffusion of analytes.[214]

A technique with the potential for imaging at higher spatial resolution is that of secondary imaging mass spectrometry (SIMS). Here, a beam of ions is fired at the sample surface to cause ionisation of the analytes. The transfer of energy from the primary ion beam to the sample causes a plume of material to be ejected from the surface and some species become ionised, secondary ions.[212,214] SIMS does not require a matrix and it can be operated in the static mode where the sample is minimally disturbed and less than 1 % of the surface removed and the dynamic mode where a large amount of the sample surface is removed. In the latter case, called NanoSIMS, ion beams can be focused to provide a lateral spatial resolution of ~50 nm.[215] In general, SIMS is considered to be a hard ionisation method compared to MALDI, causing fragmentation of analytes with larger masses. In the case of NanoSIMS, molecules are essentially atomised. Depending on the type of primary ion source, parameters such as the amount of fragmentation and the volume of sample that is ejected can be tuned to specific scientific aims.[212,214]

One key benefit of using most modes of MS is that they can be used without the need for labelling while still providing extensive chemical information. Whereas fluorescence imaging and EM typically require either treating the sample with a dye or stain, respectively, to image the specific chemical species involved, MS attains data on all molecules present given they are in high enough abundance and can be ionised.

5.3.2 The concept of imaging mass spectrometry

The idea of using images derived from mass spectra, imaging mass spectrometry (IMS), has gained increasing traction in the life sciences as mass spectrometric ionisers and analysers suitable for biology have been and continue to be developed. Another

related factor that could contribute to the spread of IMS is the aforementioned improvement in spatial resolution. When comparing MS imaging to the previously mentioned imaging techniques, the lateral spatial resolution in MS when applied for imaging have not traditionally been as good in part because of the balance required between useable detection limits, the resolution in the mass spectra, and the spatial resolution.[212,216] As with fluorescence microscopy and EM, instrumental and technical developments keep improving and commercial MS instruments that can achieve ~50 nm lateral spatial resolution exist today.[215]

The basic construction for an imaging mass spectrometer is the same as in Fig. 5.5 with an ion source, analyser and detector. The difference lies in the sampling of the sample surface. To obtain an image, several mass spectra are measured by rastering the ion source over the surface (Fig. 5.6). By selecting a certain m/z and displaying the detected intensity for each analysed spot, an image of the distribution of that particular species is obtained. This procedure can be carried out for several different chemical species and either shown alone, in different colour schemes, or in relation to an image of the sample obtained from another method (e.g. EM).[217]

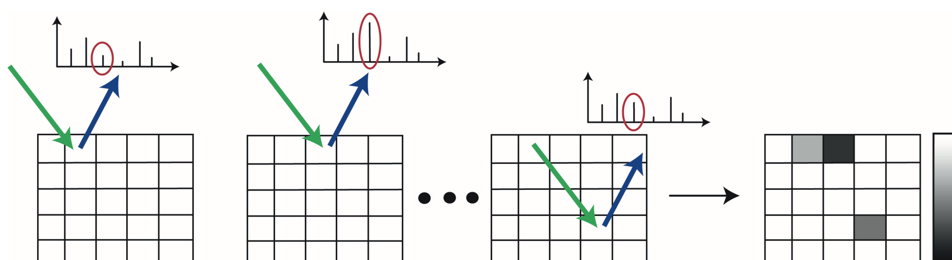

Figure 5.6 The imaging mass spectrometry principle. The selected m/z ratio here is highlighted with a red ring. In the resulting image, low intensity corresponds to lighter areas and high intensity correspond to darker areas.

5.3.3 Applications for studying cellular signalling and exocytosis

Due to the several benefits described above, several IMS methodologies have been adapted to the study of cellular communication and exocytosis. By imaging larger targets such as tissue samples, the traditionally limited spatial resolution of IMS has less

of an impact. Some examples of this application include the localisation of dietary substrates in the brain or lipid changes in the brain after traumatic injury.[218,219]

Studies of cells have also been done with IMS. As with tissue, lipid changes have been observed in both neuronal cell cultures and PC12 cells.[220,221] In addition, complementary information by IMS has been obtained for various treatments shown to cause an effect on exocytotic properties in cells. For instance, treatment with zinc and cisplatin have both been shown to alter exocytosis dynamics as measured with electrochemistry. With, IMS these treatments have both been shown to cause changes in the lipid composition of the cells, suggesting a potential mechanism involved in exocytotic regulation.[222,223]

Demonstrations of how the potential spatial resolution from IMS can contribute to the field of cellular communication are also available. A commercial instrument with lateral spatial resolution of ~50 nm, the NanoSIMS, has been used on PC12 cells following pharmacological treatment of either transmitter precursors or blockers of transmitter uptake (**paper I**). The combination of this technique with amperometry provides both quantitative data on exocytotic release as well as localisation of chemical messengers at the subcellular and even subvesicular level.[167] In these experiments, isotopically labelled dopamine was imaged between the dense core and the halo in single vesicles. The isotopic label was required as the primary ion source causes extensive fragmentation and a label facilitates differentiation between the common isotopes present in the samples and the labelled compounds introduced to the cells.

Yet another interesting combination of techniques is that of IMS and super resolution fluorescence microscopy. These complementary techniques have been used to determine the turnover rates, types, and localisation of proteins in neuronal cells.[224] Whereas the most common biological IMS measurements have been for tissue samples, the methodologies have been improving and obtaining cellular or even subcellular data are becoming more routine in the field.

Chapter 6. Summary of Papers

This thesis covers work done by me and others regarding the regulation of exocytosis in mammalian cell lines and *Drosophila* larva. By combining electrochemical techniques along with both microscopic and mass spectrometric imaging, the complex processes involved could be studied from multiple points of view. As each technique offers its own strengths and weaknesses as an analytical method, the combination provides a broader insight to exocytotic regulation than if they would have been used alone.

In **paper I,** the electrochemical methods of SCA and IVIEC are used together with TEM and IMS. The results of pharmacological treatment of PC12 cells were analysed both with high quantitative precision through SCA and IVIEC, and with high spatial precision through TEM and IMS. The cells were treated either with a dopamine precursor molecule, L-DOPA, to increase the amount of dopamine inside the vesicles, or with reserpine, a known inhibitor of VMAT that results in lower vesicular dopamine content. The effects of these drugs on vesicular dopamine content was measured both as changes in dopamine release and in total amount of dopamine stored in the vesicles. By incubating cells with isotopically labelled L-DOPA, ^{13}C-L-DOPA, the localisation of the newly stored dopamine could be determined using high resolution IMS, NanoSIMS. How enriched the vesicles were was also quantified in a semi-quantitative manner. Through the use of varying incubation times, the newly stored dopamine was observed to preferentially enter different compartments of the vesicles.

In **paper II**, PC12 cells were used again but now to investigate the effects of the breast cancer drug, tamoxifen, on exocytotic release and storage of messenger molecules in vesicles. SCA and IVIEC were applied to determine the amount of released and stored catecholamine from/in vesicles after treatment with different concentrations of tamoxifen. Interestingly, tamoxifen appeared to have an enhancing effect at low concentrations (nM) and an inhibitory effect at high concentrations (μM). Through peak analysis, these effects could be connected to changes in the fusion pore dynamics. The low concentrations slowed down exocytotic release, which in turn could add to

more catecholamine escaping the vesicle during the time the exocytotic pore was open. In addition, when measuring the amount of stored catecholamines, high concentrations of tamoxifen caused a decrease while low concentrations caused an increase. This could also contribute to the observed inhibitory or enhancing effects observed. In an attempt to deduce by which mechanism tamoxifen acted, calcium imaging using fluorescence was used. This allowed us to observe intracellular changes in calcium levels upon stimulation after tamoxifen treatment. Whereas the effects of treatment with low concentration tamoxifen hardly differed from control values, treatment with high concentrations of tamoxifen resulted in calcium levels barely detectable from the baseline.

Paper III deals with the investigation of vesicle release and storage this time in chromaffin cells. Here, the combination of SCA, IVIEC, VIEC and fluorescent calcium imaging were used to investigate the regulatory role of extracellular ATP. Incubating cells with varying concentrations of ATP followed by subsequent measurement of vesicle content and release revealed that ATP increased the fraction of vesicle content that was released. This increase occurred even though there was no alteration in vesicular catecholamine content. As in paper II, peak analysis was performed and it was concluded that the increase was due to a prolonged opening of the fusion pore as mainly the initial, rising part of the peak was affected with similar effects observed for the pre-spike feet. Here, calcium levels were also measured, but were unaltered by any ATP concentration used. To further narrow down through which pathway ATP exerted these effects, two compounds known for interfering with ATP related cellular processes were used. The first, suramin, is a non-specific purinergic blocker that can antagonise various purinergic receptors. The second, ARL-67156, antagonises ecto-ATPases that degrade extracellular ATP. As both pharmacological compounds altered the ATP induced response, purinergic receptors are likely to mediate the effects suggesting ATP can modulate exocytosis through auto receptors on the cell.

Paper IV reports the study of the effects of ATP and NE on catecholamine storage in vesicles. Knowledge about vesicular loading of extracellular NE have previously been contradictory. Due to the vital role of ATP in the maintenance of vesicular concentration and pH gradients needed to actively transport NE, we decided to investigate these

molecules together. By first incubating chromaffin cells with NE alone at different concentrations followed by quantification of vesicular content and release using SCA and IVEIC, we determined that even concentrations as high as 3.2 mM NE did not significantly increase the content inside the vesicles. Interestingly, when incubating cells with both NE and ATP at the same time, the catecholamine content inside vesicles increased in a concentration dependent manner. A corresponding increase in the number of released molecules was also observed. To test whether this effect was due to the use of ATP by the vesicle to establish a concentration and pH gradient across the vesicular membrane, a slowly hydrolysable analog of ATP was added to the cells along with NE. This did not lead to increased catecholamine storage as would be expected from an ATP analog that cannot provide energy to maintain the vesicle.

In **paper V**, the secretory activity of PC12 cells was studied with relation to presynaptic plasticity. The SCA and IVIEC techniques were used here as well as fluorescence imaging of calcium. By stimulating cells repeatedly for short periods of time, the effects of this increased activity on exocytosis were determined. Exocytotic release was measured during six consecutive stimuli and vesicular content was determined either before any stimulus, or after the third or sixth stimulus. The number of exocytotic events was observed to decrease as the cell was stimulated more as would be expected. This could in part be due to the decrease in calcium levels observed after six stimuli. Interestingly, whereas the content inside the vesicles decreased, the number of released dopamine molecules increased by contrast. After six stimuli, vesicles were estimated to release nearly all their content. This enhancement of exocytotic release was connected to longer release events as determined by peak analysis. Taken together, these results hint at a compensatory effect present in secretory cells to counter the loss of messenger molecules and usable vesicles after periods of high activity. By increasing the amount released, the outgoing chemical signal is maintained at a fairly high level suggesting a new model of plasticity and potentiation at the presynapse.

In **paper VI**, the IVIEC methodology was adapted to the more complex biological model system of *Drosophila* larva NMJ. By using genetically modified larvae that express a red fluorescent protein in octopaminergic nerve terminals, these were identified and a sharp nano-tip electrode was used to pierce the muscle into a varicosity at the NMJ. The

observed current peaks were analysed and by comparison to data from exocytotic release at larva NMJs, the fraction of exocytotic release was determined. As exocytotic release in the larva NMJ occurs either as "simple" open-and-close events or "complex" events with a flickering fusion pore, estimated percentages of release were determined separately for these two types. For simple events, only a minute fraction of transmitter was observed to be released. In the case of the complex events, the released fraction increased, but was still considerably below the fractions observed in cellular models. Due to the unexpectedly large amount of transmitters observed in the vesicle content measurements, mathematical reconstruction of release events were used to estimate the vesicular content as a complementary method. These estimates were smaller than those for the experimental IVIEC data, but on the same order of magnitude suggesting larva NMJ vesicles indeed contain much larger amounts of transmitter molecules than previously thought and only release a small fraction of this vesicular load upon stimulation. This adds further support to the idea that partial release can be a general phenomenon observed in several different species and cell types. The large vesicular load, combined with small fraction release controlled by fluctuations of the fusion pore, offers presynaptic plasticity that can be widely regulated.

Chapter 7. Concluding Remarks and Future Outlook

Throughout this thesis, studies of chemical signals in various biological systems have been described. In particular the process of exocytosis has been the major focus, as it is one main pathway for cells to communicate. Depending of what transmitter is being released, the function and dysfunction of exocytosis holds great importance and impact for any organism but perhaps for humans in particular due to their extremely complex brain and its need for proper communication to function.

Methods used for studying exocytosis and chemical signalling have been described in some detail as well as their importance to the field. Electrochemical techniques offer the possibility to characterize release and uptake of several transmitters as well as track the exocytotic process with high temporal resolution. The diversity of imaging techniques is also covered and each technique has its own strengths and weaknesses with regard to studying cellular communication.

The papers included in this thesis cover a range of methods, samples, and scientific questions. In **paper I**, imaging mass spectrometry and amperometry were used together to gain insights to how dopamine is stored inside PC12 cell vesicles. In **Paper II**, a combination of amperometry and fluorescence imaging was used to investigate the effects of the anticancer drug tamoxifen on transmitter storage and release. A similar approach was used in **paper III**, but this time focusing on the effects of ATP and how that enhances exocytosis. Experiments in **paper IV** were carried out to further investigate ATP, but in combination with added norepinephrine to reveal an increased amount stored in vesicles. Repeated stimuli were also observed to cause an enhancement of exocytotic release and this is shown in **paper V**. Finally, the amperometric method for measuring vesicular content was adapted to the *Drosophila* larva as presented in **paper VI**, and revealing a surprisingly large amount stores in these vesicles with a very small fraction released.

However, some commonalities can be found. For one, although each applied method provides insight to the question at hand, the combination of complementary techniques has been repeatedly used to shed light on a broader perspective. For instance, by gaining both spatial and temporal information about the exocytosis.

Another common feature is the high order of regulation on the exocytotic process. This regulation can be induced either by external effects, such as application of a pharmacological reagent, or by intrinsic effects such as the secretory activity of the cell. By using partial release, cells can regulate the strength of the chemical signal they send out and this phenomenon has been observed in both mammalian cell cultures and in invertebrate neurons. Further studies are needed to establish partial release in human cells as well as the level and mechanisms involved.

However, the implied conservation of a regulatory pathway and the fact that both external and intrinsic factors have an effect brings great promise to the future of this field. By learning more about what external factors affect partial release we can perhaps guide the development of new drugs targeting erroneous chemical signalling. As discussed previously, many diseases involve dysfunctional signalling between cells making the opportunity to target new processes in order to ameliorate disease progress is of tremendous value. On the other hand, knowledge concerning the intrinsic factors can provide further evidence into how plasticity takes place in various cells. Understanding this could be key to fully grasp various mechanisms about the human mind and memory.

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References

(1) Lucic, V., Yang, T., Schweikert, G., Förster, F., and Baumeister, W. (2005) Morphological Characterization of Molecular Complexes Present in the Synaptic Cleft. *Structure 13*, 423–434.
(2) Zuber, B., Nikonenko, I., Klauser, P., Muller, D., and Dubochet, J. (2005) The Mammalian Central Nervous Synaptic Cleft Contains a High Density of Periodically Organized Complexes. *Proc. Natl. Acad. Sci. U. S. A. 102*, 19192–19197.
(3) Ludwig, M., and Pittman, Q. J. (2003) Talking Back: Dendritic Neurotransmitter Release. *Trends Neurosci. 26*, 255–261.
(4) Rozental, R., Giaume, C., and Spray, D. C. (2000) Gap Junctions in the Nervous System. *Brain Res. Rev. 32*, 11–15.
(5) Bernardini, G. L., Gu, X., Viscardi, E., and German, D. C. (1991) Amphetamine-Induced and Spontaneous Release of Dopamine from A9 and A10 Cell Dendrites: An *In Vitro* Electrophysiological Study in the Mouse. *J. Neural Transm. 84*, 183–193.
(6) Zagorodnyuk, V. P., Gregory, S., Costa, M., Brookes, S. J. H., Tramontana, M., Giuliani, S., and Maggi, C. A. (2009) Spontaneous Release of Acetylcholine from Autonomic Nerves in the Bladder. *Br. J. Pharmacol. 157*, 607–619.
(7) Thorn, P., Zorec, R., Rettig, J., and Keating, D. J. (2016) Exocytosis in Non-Neuronal Cells. *J. Neurochem. 137*, 849–859.
(8) Low, J. T., Zavortink, M., Mitchell, J. M., Gan, W. J., Do, O. H., Schwiening, C. J., Gaisano, H. Y., and Thorn, P. (2014) Insulin Secretion from Beta Cells in Intact Mouse Islets is Targeted Towards the Vasculature. *Diabetologia 57*, 1655–1663.
(9) Blakely, R. D., and Edwards, R. H. (2012) Vesicular and Plasma Membrane Transporters for Neurotransmitters. *Cold Spring Harb. Perspect. Biol. 4*, 1–24.
(10) Rudnick, G., and Clark, J. (1993) From Synapse to Vesicle: The Reuptake and Storage of Biogenic Amine Neurotransmitters. *Biochim. Biophys. Acta 1144*, 249–263.
(11) Staal, R. G. W., Mosharov, E. V., and Sulzer, D. (2004) Dopamine Neurons Release Transmitter via a Flickering Fusion Pore. *Nat. Neurosci. 7*, 341–346.
(12) Leszczyszyn, D. J., Jankowski, J. A., Viveross, H., Diliberto, E. J., Nears, J. A., and Wightman, R. M. (1990) Nicotinic Receptor-Mediated Catecholamine Secretion from Individual Chromaffin Cells. *J. Biol. Chem. 265*, 14736–14737.
(13) Mahapatra, N. R. (2008) Catestatin is a Novel Endogenous Peptide that Regulates Cardiac Function and Blood Pressure. *Cardiovasc. Res. 80*, 330–338.
(14) Abraham, W. C., and Bear, M. F. (1996) Metaplasticity: The Plasticity of Synaptic Plasticity. *Trends Neurosci. 19*, 126–130.

(15) Lamprecht, R., and LeDoux, J. (2004) Structural Plasticity and Memory. *Nat. Rev. Neurosci. 5*, 45–54.
(16) Martin, S. J., Grimwood, P. D., and Morris, R. G. M. (2000) Synaptic Plasticity and Memory: An Evaluation of the Hypothesis. *Annu. Rev. Neurosci. 23*, 649–711.
(17) Murthy, V. N. (1998) Synaptic Plasticity: Step-Wise Strengthening. *Curr. Biol. 8*, R650–R653.
(18) Sola, E., Prestori, F., Rossi, P., Taglietti, V., and D'Angelo, E. (2004) Increased Neurotransmitter Release during Long-term Potentiation at Mossy Fibre-Granule Cell Synapses in Rat Cerebellum. *J. Physiol. 557*, 843–861.
(19) Bliss, T. V. P., and Collingridge, G. L. (2013) Expression of NMDA Receptor-dependent LTP in the Hippocampus: Bridging the Divide. *Mol. Brain 6*.
(20) Sharma, N., Classen, J., and Cohen, L. G. (2013) Neural Plasticity and its Constribution to Functional Recovery. *Handb Clin Neurol 110*, 3–12.
(21) Kandel, E. R., Schwartz, J. H., Jessel, T. M., Siegelbaum, S. A., and Hudspeth, A. J. (Eds.). (2013) Principles of Neural Science 5th edition. The McGraw-Hill Companies, Inc.
(22) Sala, F., Nistri, A., and Criado, M. (2008) Nicotinic Acetylcholine Receptors of Adrenal Chromaffin Cells. *Acta Physiol. 192*, 203–212.
(23) Zerby, S. E., and Ewing, A. G. (2002) The Latency of Exocytosis Varies with the Mechanism of Stimulated Release in PC12 Cells. *J. Neurochem. 66*, 651–657.
(24) García, A. G., García-De-Diego, A. M., Gandía, L., Borges, R., and García-Sancho, J. (2006) Calcium Signaling and Exocytosis in Adrenal Chromaffin Cells. *Physiol. Rev. 86*, 1093–1131.
(25) Lorentz, A., Baumann, A., Vitte, J., and Blank, U. (2012) The SNARE Machinery in Mast Cell Secretion. *Front. Immunol. 3*, 1–17.
(26) Neher, E., and Sakaba, T. (2008) Multiple Roles of Calcium Ions in the Regulation of Neurotransmitter Release. *Neuron 59*, 861–872.
(27) Verkhratsky, A. J., and Petersen, O. H. (1998) Neuronal Calcium Stores. *Cell Calcium 24*, 333–343.
(28) SantoDomingo, J., Vay, L., Camacho, M., Hernández-SanMiguel, E., Fonteriz, R. I., Lobatón, C. D., Montero, M., Moreno, A., and Alvarez, J. (2008) Calcium Dynamics in Bovine Adrenal Medulla Chromaffin Cell Secretory Granules. *Eur. J. Neurosci. 28*, 1265–1274.
(29) Arundine, M., and Tymianski, M. (2003) Molecular Mechanisms of Calcium-Dependent Neurodegeneration in Excitotoxicity. *Cell Calcium 34*, 325–337.
(30) Nadim, F., and Bucher, D. (2014) Neuromodulation of Neurons and Synapses.

Curr. Opin. Neurobiol. December, 48–56.
(31) Cavallotti, C., Mancone, M., Bruzzone, P., Sabbatini, M., and Mignini, F. (2010) Dopamine Receptor Subtypes in the Native Human Heart. *Hear. Vessel. 25*, 432–437.
(32) Ozono, R., O'Connell, D. P., Wang, Z. Q., Moore, A. F., Sanada, H., Felder, R. A., and Carey, R. M. (1997) Localization of the Dopamine D1 Receptor Protein in the Human Heart and Kidney. *Hypertension 30*, 725–729.
(33) Zhang, Y., Zheng, R., Meng, X., Wang, L., Liu, L., and Gao, Y. (2015) Pancreatic Endocrine Effects of Dopamine Receptors in Human Islet Cells. *Pancreas 44*, 925–929.
(34) Wise, R. A. (2004) Dopamine, Learning and Motivation. *Nat. Rev. Neurosci. 5*, 483–494.
(35) Monti, J. M., and Monti, D. (2007) The Involvement of Dopamine in the Modulation of Sleep and Waking. *Sleep Med. Rev. 11*, 113–133.
(36) Kuhar, M. J., Ritz, M. C., and Boja, J. W. (1991) The Dopamine Hypothesis of the Reinforcing Properties of Cocaine. *Trends Neurosci. 14*, 299–302.
(37) Nutt, D. J., Lingford-Hughes, A., Erritzoe, D., and Stokes, P. R. A. (2015) The Dopamine Theory of Addiction: 40 Years of Highs and Lows. *Nat. Rev. Neurosci. 16*, 305–312.
(38) Lyon, G. J., Abi-Dargham, A., Moore, H., Lieberman, J. A., Javitch, J. A., and Sulzer, D. (2011) Presynaptic Regulation of Dopamine Transmission in Schizophrenia. *Schizophr. Bull. 37*, 108–117.
(39) Volkow, N. D., Wang, G., Fowler, J. S., Logan, J., Gerasimov, M., Maynard, L., Ding, Y., Gatley, S. J., Gifford, A., and Franceschi, D. (2001) Therapeutic Doses of Oral Methylphenidate Significantly Increase Extracellular Dopamine in the Human Brain. *J. Neurosci. 21*, RC121.
(40) Troger, J., Kirchmair, R., Marksteiner, J., Seidl, C. V, Fischer-Colbrie, R., Saria, A., and Winkler, H. (1994) Release of Secretoneurin and Noradrenaline from Hypothalamic Slices and its Differential Inhibition by Calcium Channel Blockers. *Naunyn. Schmiedebergs. Arch. Pharmacol. 349*, 565–569.
(41) Kennedy, B. P., Mahata, S. K., O'Connor, D. T., and Ziegler, M. G. (1998) Mechanism of Cardiovascular Actions of the Chromogranin A Fragment Catestatin *In Vivo*. *Peptides 19*, 1241–1248.
(42) Wiedermann, C. J. (2000) Secretoneurin: A Functional Neuropeptide in Health and Disease. *Peptides 21*, 1289–1298.
(43) Floras, J. S. (1992) Epinephrine and the Genesis of Hypertension. *Hypertension 19*, 1–18.
(44) Moret, C., and Briley, M. (2011) The Importance of Norepinephrine in

Depression. *Neuropsychiatr. Dis. Treat. 7*, 9–13.
(45) Cooper, S. E., and Venton, B. J. (2009) Fast-scan Cyclic Voltammetry for the Detection of Tyramine and Octopamine. *Anal. Bioanal. Chem. 394*, 329–336.
(46) Grygoruk, A., Chen, A., Martin, C. A., Lawal, H. O., Fei, H., Gutierrez, G., Biedermann, T., Najibi, R., Hadi, R., Chouhan, A. K., Murphy, N. P., Schweizer, F. E., Macleod, G. T., Maidment, N. T., and Krantz, D. E. (2014) The Redistribution of Drosophila Vesicular Monoamine Transporter Mutants from Synaptic Vesicles to Large Dense-Core Vesicles Impairs Amine-Dependent Behaviors. *J. Neurosci. 34*, 6924–6937.
(47) Berry, M. D. (2004) Mammalian Central Nervous System Trace Amines. Pharmacologic Amphetamines, Physiologic Neuromodulators. *J. Neurochem. 90*, 257–271.
(48) Jahn, R., and Sudhof, T. C. (1993) Synaptic Vesicle Traffic: Rush Hour in the Nerve Terminal. *J. Neurochem. 61*, 12–21.
(49) Schikorski, T., and Stevens, C. F. (1997) Quantitative Ultrastructural Analysis of Hippocampal Excitatory Synapses. *J. Neurosci. 17*, 5858–5867.
(50) Huttner, W. B., Gerdes, H.-H., and Rosa, P. (1991) The Granin (Secretogranin-Chromogranin) Family. *Trends Biochem. Sci. 16*, 27–30.
(51) Wiedenmann, B., and Huttner, W. B. (1989) Synaptophysin and Chromogranins/Secretogranins Widespread Constituents of Distinct Types of Neuroendocrine Vesicles and New Tools in Tumor Diagnosis. *Virchows Arch. B Cell Pathol. Incl. Mol. Pathol. 58*, 95–121.
(52) Videen, J. S., Mezger, M. S., Chang, Y., and O'Connor, D. T. (1992) Calcium and Catecholamine Interactions with Adrenal Chromogranins. *J. Biol. Chem. 267*, 3066–3073.
(53) Yoo, S. H., Albanesi, J. P., and Jameson, D. M. (1990) Fluorescence Studies of Nucleotide Interactions with Bovine Adrenal Chromogranin A. *Biochim. Biophys. Acta 1040*, 66–70.
(54) Helle, K. B., Reed, R. K., Pihl, K. E., and Serck-Hanssen, G. (1985) Osmotic Properties of the Chromogranins and Relation to Osmotic Pressure in Catecholamine Storage Granules. *Acta Physiol. Scand. 123*, 21–33.
(55) Montesinos, M. S., Machado, J. D., Camacho, M., Diaz, J., Morales, Y. G., Alvarez de la Rosa, D., Carmona, E., Castaneyra, A., Viveros, O. H., O'Connor, D. T., Mahata, S. K., and Borges, R. (2008) The Crucial Role of Chromogranins in Storage and Exocytosis Revealed Using Chromaffin Cells from Chromogranin A Null Mouse. *J. Neurosci. 28*, 3350–3358.

(56) Domínguez, N., Estévez-Herrera, J., Pardo, M. R., Pereda, D., Machado, J. D., and Borges, R. (2012) The Functional Role of Chromogranins in Exocytosis. *J. Mol. Neurosci. 48*, 317–322.
(57) Diaz-Vera, J., Morales, Y. G., Hernandez-Fernaud, J. R., Camacho, M., Montesinos, M. S., Calegari, F., Huttner, W. B., Borges, R., and Machado, J. D. (2010) Chromogranin B Gene Ablation Reduces the Catecholamine Cargo and Decelerates Exocytosis in Chromaffin Secretory Vesicles. *J. Neurosci. 30*, 950–957.
(58) Oleinick, A., Hu, R., Ren, B., Tian, Z.-Q., Svir, I., and Amatore, C. (2016) Theoretical Model of Neurotransmitter Release during *In Vivo* Vesicular Exocytosis Based on a Grainy Biphasic Nano-Structuration of Chromogranins within Dense Core Matrixes. *J. Electrochem. Soc. 163*, H3014–H3024.
(59) Jia, X. X., Gorczyca, M., and Budnik, V. (1993) Ultrastructure of Neuromuscular Junctions in *Drosophila* Comparison of Wild Type and Mutants with Increased Excitability. *J. Neurobiol. 24*, 1025–1044.
(60) Dean, P. M. (1973) Ultrastructural Morphometry of the Pancreatic β-Cell. *Diabetologia 9*, 115–119.
(61) Plattner, H., Artalejo, A. R., and Neher, E. (1997) Ultrastructural Organization of Bovine Chromaffin Cell Cortex - Analysis by Cryofixation and Morphometry of Aspects Pertinent to Exocytosis. *J. Cell Biol. 139*, 1709–1717.
(62) Atwood, H. L., Govind, C. K., and Wu, C.-F. (1993) Differential Ultrastructure of Synaptic Terminals on Ventral Longitudinal Abdominal Muscles in *Drosophila* Larvae. *J. Neurobiol. 24*, 1008–1024.
(63) Forgac, M. (2007) Vacuolar ATPases: Rotary Proton Pumps in Physiology and Pathophysiology. *Nat. Rev. Mol. Cell Biol. 8*, 917–929.
(64) Wimalasena, K. (2011) Vesicular Monoamine Transporters: Structure-Function, Pharmacology, and Medicinal Chemistry. *Med. Res. Rev. 31*, 483–519.
(65) Albillos, A., Dernick, G., Horstmann, H., Almers, W., Alvarez de Toledo, G., and Lindau, M. (1997) The Exocytotic Event in Chromaffin Cells Revealed by Patch Amperometry. *Nature 389*, 509–512.
(66) Sawada, K., Echigo, N., Juge, N., Miyaji, T., Otsuka, M., Omote, H., Yamamoto, A., and Moriyama, Y. (2008) Identification of a Vesicular Nucleotide Transporter. *Proc. Natl. Acad. Sci. 105*, 5683–5686.
(67) Burnstock, G., and Verkhratsky, A. (2009) Evolutionary Origins of the Purinergic Signalling System. *Acta Physiol. 195*, 415–447.
(68) Verhage, M., and Sørensen, J. B. (2008) Vesicle Docking in Regulated Exocytosis. *Traffic 9*, 1414–1424.

(69) Klenchin, V. A., and Martin, T. F. J. (2000) Priming in Exocytosis: Attaining Fusion-Competence after Vesicle Docking. *Biochimie 82*, 399–407.
(70) Matos, M. F., Mukherjee, K., Chen, X., Rizo, J., and Südhof, T. C. (2003) Evidence for SNARE Zippering during Ca^{2+}-Triggered Exocytosis in PC12 Cells. *Neuropharmacology 45*, 777–786.
(71) Zhang, Z., Wu, Y., Wang, Z., Dunning, F. M., Rehfuss, J., Ramanan, D., Chapman, E. R., and Jackson, M. B. (2011) Release Mode of Large and Small Dense-Core Vesicles Specified by Different Synaptotagmin Isoforms in PC12 Cells. *Mol. Biol. Cell 22*, 2324–2336.
(72) Söllner, T., Whiteheart, S. W., Brunner, M., Erdjument-Bromage, H., Geromanos, S., Tempst, P., and Rothman, J. E. (1993) SNAP Receptors Implicated in Vesicle Targeting and Fusion. *Nature 362*, 318–324.
(73) Brunger, A. T., Fiebig, K. M., Rice, L. M., and Pollock, E. (1999) Folding Intermediates of SNARE Complex Assembly. *Nat. Struct. Biol. 6*, 117–123.
(74) Trouillon, R., and Ewing, A. G. (2014) Actin Controls the Vesicular Fraction of Dopamine Released During Extended Kiss and Run Exocytosis. *ACS Chem. Biol. 9*, 812–820.
(75) Anantharam, A., Bittner, M. A., Aikman, R. L., Stuenkel, E. L., Schmid, S. L., Axelrod, D., and Holz, R. W. (2011) A New Role for the Dynamin GTPase in the Regulation of Fusion Pore Expansion. *Mol. Biol. Cell 22*, 1907–1918.
(76) Jackson, J., Papadopulos, A., Meunier, F. A., Mccluskey, A., Robinson, P. J., and Keating, D. J. (2015) Small Molecules Demonstrate the Role of Dynamin as a Bi-Directional Regulator of the Exocytosis Fusion Pore and Vesicle Release. *Mol. Psychiatry 20*, 810–819.
(77) Zucker, R. S., Kullmann, D. M., and Kaeser, P. S. (2014) Release of Neurotransmitters. *From Mol. to Networks An Introd. to Cell. Mol. Neurosci.* (Byrne, J. H., Hiedelberger, R., and Waxham, M. N., Eds.) 3rd edition. Elsevier Inc.
(78) Ren, L., Mellander, L. J., Keighron, J., Cans, A.-S., Kurczy, M. E., Svir, I., Oleinick, A., Amatore, C., and Ewing, A. G. (2016) The Evidence for Open and Closed Exocytosis as the Primary Release Mechanism. *Q. Rev. Biophys. 49*, e12.
(79) Phan, N. T. N., Li, X., and Ewing, A. G. (2017) Measuring Synaptic Vesicles Using Cellular Electrochemistry and Nanoscale Molecular Imaging. *Nat. Rev. 1*, 0048.
(80) Südhof, T. C. (2004) The Synaptic Vesicle Cycle. *Annu. Rev. Neurosci. 27*, 509–547.
(81) Rizzoli, S. O. (2014) Synaptic Vesicle Recycling: Steps and Principles. *EMBO J. 33*, 788–822.

(82) Schneggenburger Ralf, and Neher Erwin. (2000) Intracellular Calcium Dependence of Transmitter Release Rates at a Fast Central Synapse. *Nature 406*, 889–893.
(83) Elhamdani, A., Palfrey, H. C., and Artalejo, C. R. (2001) Quantal Size is Dependent on Stimulation Frequency and Calcium Entry in Calf Chromaffin Cells. *Neuron 31*, 819–830.
(84) Dunevall, J., Fathali, H., Najafinobar, N., Lovric, J., Wigström, J., Cans, A. S., and Ewing, A. G. (2015) Characterizing the Catecholamine Content of Single Mammalian Vesicles by Collision-Adsorption Events at an Electrode. *J. Am. Chem. Soc. 137*, 4344–4346.
(85) Li, X., Majdi, S., Dunevall, J., Fathali, H., and Ewing, A. G. (2015) Quantitative Measurement of Transmitters in Individual Vesicles in the Cytoplasm of Single Cells with Nanotip Electrodes. *Angew. Chemie - Int. Ed. 54*, 11978–11982.
(86) Ren, L., Pour, M. D., Majdi, S., Li, X., Malmberg, P., and Ewing, A. G. (2017) Zinc Regulates Chemical-Transmitter Storage in Nanometer Vesicles and Exocytosis Dynamics as Measured by Amperometry. *Angew. Chemie - Int. Ed. 56*, 4970–4975.
(87) Majdi, S., Najafinobar, N., Dunevall, J., Lovric, J., and Ewing, A. G. (2017) DMSO Chemically Alters Cell Membranes to Slow Exocytosis and Increase the Fraction of Partial Transmitter Released. *ChemBioChem 18*, 1898–1902.
(88) Zhu, W., Gu, C., Dunevall, J., Ren, L., Zhou, X., and Ewing, A. G. (2019) Combined Amperometry and Electrochemical Cytometry Reveal Differential Effects of Cocaine and Methylphenidate on Exocytosis and the Fraction of Chemical Release. *Angew. Chemie - Int. Ed. 58*, 4238–4242.
(89) Najafinobar, N., Mellander, L. J., Kurczy, M. E., Dunevall, J., Angerer, T. B., Fletcher, J. S., and Cans, A. S. (2016) Cholesterol Alters the Dynamics of Release in Protein Independent Cell Models for Exocytosis. *Sci. Rep. 6*, 1–10.
(90) Helle, K. B., Reed, R. K., Pihl, K. E., and Serck-Hanssen, G. (1985) Osmotic properties of the chromogranins and relation to osmotic pressure in catecholamine storage granules. *Acta Physiol. Scand. 123*, 21–33.
(91) Pothos, E., Desmond, M., and Sulzer, D. (2002) l-3,4-Dihydroxyphenylalanine Increases the Quantal Size of Exocytotic Dopamine Release In Vitro. *J. Neurochem. 66*, 629–636.
(92) Trommershäuser, J., Schneggenburger, R., Zippelius, A., and Neher, E. (2003) Heterogeneous Presynaptic Release Probabilities: Functional Relevance for Short-Term Plasticity. *Biophys. J. 84*, 1563–1579.
(93) Greene, L. A., and Tischler, A. S. (1976) Establishment of a Noradrenergic Clonal

Line of Rat Adrenal Pheochromocytoma Cells which Respond to Nerve Growth Factor. *Proc. Natl. Acad. Sci. 73*, 2424–2428.
(94) Du, M., Flanigan, V., and Ma, Y. (2004) Simultaneous Determination of Polyamines and Catecholamines in PC-12 Tumor Cell Extracts by Capillary Electrophoresis with Laser-Induced Fluorescence Detection. *Electrophoresis 25*, 1496–1502.
(95) Kumar, G. K., Overholt, J. L., Bright, G. R., Hui, K. Y., Lu, H., Gratzl, M., and Prabhakar, N. R. (1998) Release of Dopamine and Norepinephrine by Hypoxia from PC-12 Cells. *Am. J. Physiol. - Cell Physiol. 274*, C1592–C1600.
(96) Das, P. C., McElroy, W. K., and Cooper, R. L. (2001) Alteration of Catecholamines in Pheochromocytoma (PC12) Cells *In Vitro* by the Metabolites of Chlorotriazine Herbicide. *Toxicol. Sci. 59*, 127–137.
(97) Chen, T. K., Luo, G., and Ewing, A. G. (1994) Amperometric Monitoring of Stimulated Catecholamine Release from Rat Pheochromocytoma (PC12) Cells at the Zeptomole Level. *Anal. Chem. 66*, 3031–3035.
(98) Westerink, R. H. S., and Ewing, A. G. (2008) The PC12 Cell as Model for Neurosecretion. *Acta Physiol. 192*, 273–285.
(99) DeBerardinis, R. J., and Chandel, N. S. (2016) Fundamentals of Cancer Metabolism. *Sci. Adv. 2*, e1600200.
(100) Shoji-Kasai, Y., Morishima, M., Kuwahara, R., Kondo, S., Itakura, M., and Takahashi, M. (2001) Establishment of Variant PC12 Subclones Deficient in Stimulation-Secretion Coupling. *Biochim. Biophys. Acta 1499*, 180–190.
(101) Zerby, S. E., and Ewing, A. G. (1996) Electrochemical Monitoring of Individual Exocytotic Events from the Varicosities of Differentiated PC12 Cells. *Brain Res. 712*, 1–10.
(102) Stallcup, W. B. (1979) Sodium and Calcium Fluxes in a Clonal Nerve Cell Line. *J. Physiol. 286*, 525–540.
(103) Zhu, W. H., Conforti, L., and Millhorn, D. E. (1997) Expression of Dopamine D2 Receptor in PC-12 Cells and Regulation of Membrane Conductances by Dopamine. *Am. J. Physiol. Cell Physiol. 273*, C1143–C1150.
(104) Kim, W. K., and Rabin, R. A. (1994) Characterization of the Purinergic P2 Receptors in PC12 Cells: Evidence for a Novel Subtype. *J. Biol. Chem. 269*, 6471–6477.
(105) Williams, N. G., Zhong, H., and Minneman, K. P. C. N.-C. (1998) Differential Coupling of Alpha1-, Alpha2-, and Beta-Adrenergic Receptors to Mitogen-Activated Protein Kinase Pathways and Differentiation in Transfected PC12 Cells. *J. Biol. Chem. 273*, 24624–24632.

(106) Albillos, A., Artalejo, A. R., López, M. G., Gandía, L., García, A. G., and Carbone, E. (1994) Calcium Channel Subtypes in Cat Chromaffin Cells. *J. Physiol. 477*, 197–213.
(107) Cortez, V., Santana, M., Marques, A. P., Mota, A., Rosmaninho-Salgado, J., and Cavadas, C. (2012) Regulation of Catecholamine Release in Human Adrenal Chromaffin Cells by β-Adrenoceptors. *Neurochem. Int. 60*, 387–393.
(108) Liu, M., Dunn, P. M., King, B. F., and Burnstock, G. (1999) Rat Chromaffin Cells Lack P2X Receptors While Those of the Guinea-Pig Express a P2X Receptor with Novel Pharmacology. *Br. J. Pharmacol. 128*, 61–68.
(109) Lovell, P. V, James, D. G., and McCobb, D. P. (2000) Bovine Versus Rat Adrenal Chromaffin Cells: Big Differences in BK Potassium Channel Properties. *J. Neurophysiol. 83*, 3277–3286.
(110) Lovrić, J., Malmberg, P., Johansson, B. R., Fletcher, J. S., and Ewing, A. G. (2016) Multimodal Imaging of Chemically Fixed Cells in Preparation for NanoSIMS. *Anal. Chem. 88*, 8841–8848.
(111) Bartolomucci, A., Possenti, R., Mahata, S. K., Fischer-Colbrie, R., Loh, Y. P., and Salton, S. R. J. (2011) The Extended Granin Family: Structure, Function, and Biomedical Implications. *Endocr. Rev. 32*, 755–797.
(112) von Rüden, L., García, A. G., and López, M. G. (1993) The Mechanism of Ba^{2+} -Induced Exocytosis From Single Chromaffin Cells. *FEBS Lett. 336*, 48–52.
(113) Heldman, E., Levine, M., Raveh, L., and Pollard, H. B. (1989) Barium Ions Enter Chromaffin Cells via Voltage-Dependent Calcium Channels and Induce Secretion by a Mechanism Independent of Calcium. *J. Biol. Chem. 264*, 7914–7920.
(114) Tomsig, J. L., and Suszkiw, J. B. (2002) Metal Selectivity of Exocytosis in α-Toxin-Permeabilized Bovine Chromaffin Cells. *J. Neurochem. 66*, 644–650.
(115) Stocker, H., and Gallant, P. (2008) Getting Started: An Overview on Raising and Handling *Drosophila*. *Methods Mol. Biol. 420*, 27–44.
(116) Reiter, L. T., Potocki, L., Chien, S., Gribskov, M., and Bier, E. (2001) A Systematic Analysis of Human Disease-Associated Gene Sequences In *Drosophila* melanogaster. *Genome Res. 11*, 1114–1125.
(117) Rubin, G. M., Yandell, M. D., Wortman, J. R., Gabor Miklos, G. L., Nelson, C. R., Hariharan, I. K., Fortini, M. E., Li, P. W., Apweiler, R., Fleischmann, W., Cherry, J. M., Henikoff, S., Skupski, M. P., Misra, S., Ashburner, M., Birney, E., Boguski, M. S., Brody, T., Brokstein, P., Celniker, S. E., Chervitz, S. A., Coates, D., Cravchik, A., Gabrielian, A., Galle, R. F., Gelbart, W. M., George, R. A., Goldstein, L. S., Gong, F., Guan, P., Harris, N. L., Hay, B. A., Hoskins, R. A., Li, J., Li, Z., Hynes, R. O., Jones, S. J. M., Kuehl, P. M., Lemaitre, B., Littleton, J. T., Morrison, D. K., Mungall, C., O'Farrell,

P. H., Pickeral, O. K., Shue, C., Vosshall, L. B., Zhang, J., Zhao, Q., Zheng, X. H., Zhong, F., Zhong, W., Gibbs, R., Venter, C. J., Adams, M. D., and Lewis, S. (2000) Comparative Genomics of the Eukaryotes. *Science (80-.). 287*, 2204–2215.
(118) O'Kane, C. J. (2011) *Drosophila* as a Model Organism for the Study of Neuropsychiatric Disorders. *Mol. Funct. Model. Neuropsychiatry* 37–60.
(119) Scott, K., Brady, R., Cravchik, A., Morozov, P., Rzhetsky, A., Zuker, C., and Axel, R. (2001) A Chemosensory Gene Family Encoding Candidate Gustatory and Olfactory Receptors in *Drosophila*. *Cell 104*, 661–673.
(120) Herculano-Houzel, S. (2009) The Human Brain in Numbers: A Linearly Scaled-Up Primate Brain. *Front. Hum. Neurosci. 3*, 1–11.
(121) Denno, M. E., Privman, E., and Venton, B. J. (2015) Analysis of Neurotransmitter Tissue Content of *Drosophila* melanogaster in Different Life Stages. *ACS Chem. Neurosci. 6*, 117–123.
(122) Yasuyama, K., Kitamoto, T., and Salvaterra, P. M. (1995) Localization of Choline Acetyltransferase-Expresing Neurons in the Larval Visual System of *Drosophila* melanogaster. *Cell Tissue Res. 282*, 193–202.
(123) Daniels, R. W., Gelfand, M. V., Collins, C. A., and DiAntonio, A. (2008) Visualizing Glutamatergic Cell Bodies and Synapses in *Drosophila* Larval and Adult CNS. *J. Comp. Neurol. 508*, 131–152.
(124) Monastirioti, M., Gorczyca, M., Rapus, J., Eckert, M., White, K., and Budnik, V. (1995) Octopamine Immunoreactivity in the Fruit Fly *Drosophila melanogaster*. *J. Comp. Neurol. 356*, 37–54.
(125) Johansen, J., Halpern, M. E., Johansen, K. M., and Keshishian, H. (1989) Stereotypic Morphology of Glutamatergic Synapses on Identified Muscle Cells of Drosophila Larvae. *J. Neurosci. 9*, 710–725.
(126) Gorczyca, M., Augart, C., and Budnik, V. (1993) Insulin-like receptor and Insulin-like Peptide are Localized at Neuromuscular Junctions in *Drosophila*. *J. Neurosci. 13*, 3692–3704.
(127) Denno, M. E., Privman, E., and Venton, B. J. (2015) Analysis of Neurotransmitter Tissue Content of *Drosophila melanogaster* in Different Life Stages. *ACS Chem. Neurosci. 6*, 117–123.
(128) Pollack, I., and Hofbauer, A. (1991) Histamine-like Immunoreactivity in the Visual System and Brain of *Drosophila melanogaster*. *Cell Tissue Res. 266*, 391–398.
(129) Chiang, A. S., Lin, C. Y., Chuang, C. C., Chang, H. M., Hsieh, C. H., Yeh, C. W., Shih, C. T., Wu, J. J., Wang, G. T., Chen, Y. C., Wu, C. C., Chen, G. Y., Ching, Y. T., Lee, P. C., Lin, C. Y., Lin, H. H., Wu, C. C., Hsu, H. W., Huang, Y. A., Chen, J. Y.,

Chiang, H. J., Lu, C. F., Ni, R. F., Yeh, C. Y., and Hwang, J. K. (2011) Three-Dimensional Reconstruction of Brain-Wide Wiring Networks in *Drosophila* at Single-Cell Resolution. *Curr. Biol. 21*, 1–11.
(130) Yamagata, N., Ichinose, T., Aso, Y., Plaçais, P.-Y., Friedrich, A. B., Sima, R. J., Preat, T., Rubin, G. M., and Tanimoto, H. (2014) Distinct Dopamine Neurons Mediate Reward Signals for Short- and Long-Term Memories. *Proc. Natl. Acad. Sci. U. S. A. 112*, 578–83.
(131) Grover, D., Katsuki, T., and Greenspan, R. J. (2016) Flyception: Imaging Brain Activity in Freely Walking Fruit Flies. *Nat. Methods 13*, 569–572.
(132) Brand, A. H., and Perrimon, N. (1993) Targeted Gene Expression as a Means of Altering Cell Fates and Generating Dominant Phenotypes. *Development 118*, 401–415.
(133) Nagel, G., Szellas, T., Huhn, W., Kateriya, S., Adeishvili, N., Berthold, P., Ollig, D., Hegemann, P., and Bamberg, E. (2003) Channelrhodopsin-2, A directly Light-Gated Cation-Selective Membrane Channel. *Proc. Natl. Acad. Sci. U. S. A. 100*, 13940–13945.
(134) Nagel, G., Ollig, D., Fuhrmann, M., Kateriya, S., Musti, A. M., Bamberg, E., and Hegemann, P. (2002) Channelrhodopsin-1: A Light-Gated Proton Channel in Green Algae. *Science (80-.). 296*, 2395–2398.
(135) Lin, J. Y., Lin, M. Z., Steinbach, P., and Tsien, R. Y. (2009) Characterization of Engineered Channelrhodopsin Variants with Improved Properties and Kinetics. *Biophys. J. 96*, 1803–1814.
(136) Gunaydin, L. A., Yizhar, O., Berndt, A., Sohal, V. S., Deisseroth, K., and Hegemann, P. (2010) Ultrafast Optogenetic Control. *Nat. Neurosci. 13*, 387–392.
(137) Majdi, S., Berglund, E. C., Dunevall, J., Oleinick, A. I., Amatore, C., Krantz, D. E., and Ewing, A. G. (2015) Electrochemical Measurements of Optogenetically Stimulated Quantal Amine Release from Single Nerve Cell Varicosities in *Drosophila* Larvae. *Angew. Chemie - Int. Ed. 54*, 13609–13612.
(138) Michael, A. C., and Borland, L. M. (Eds.). (2007) Electrochemical Methods for Neuroscience. CRC Press.
(139) Sassolas, A., Blum, L. J., and Leca-Bouvier, B. D. (2012) Immobilization Strategies to Develop Enzymatic Biosensors. *Biotechnol. Adv. 30*, 489–511.
(140) Bard, A. J., and Faulkner, L. R. (2001) Electrochemical Methods: Fundamentals and Applications 2nd edition. John Wiley & Sons, Inc.
(141) Wang, J. (2006) Analytical Electrochemistry (Edition, 3rd, Ed.). John Wiley & Sons, Inc.
(142) Samaranayake, S., Abdalla, A., Robke, R., Wood, K. M., Zeqja, A., and Hashemi,

P. (2015) *In Vivo* Histamine Voltammetry in the Mouse Premammillary Nucleus. *Analyst 140*, 3759–3765.
(143) Heien, M. L. A. V., Phillips, P. E. M., Stuber, G. D., Seipel, A. T., and Wightman, R. M. (2003) Overoxidation of Carbon-Fiber Microelectrodes Enhances Dopamine Adsorption and Increases Sensitivity. *Analyst 128*, 1413–1419.
(144) Hashemi, P., Dankoski, E. C., Petrovic, J., Keithley, R. B., and Wightman, R. M. (2009) Voltammetric Detection of 5-Hydroxytryptamine Release in the Rat Brain. *Anal. Chem. 81*, 9462–9471.
(145) Pyakurel, P., Privman, E., and Venton, B. J. (2016) Fast-Scan Cyclic Voltammetry (FSCV) Detection of Endogenous Octopamine in *Drosophila melanogaster* Ventral Nerve Cord. *ACS Chem. Neurosci. 7*, 1112–1119.
(146) Covey, D. P., and Cheer, J. F. (2019) Accumbal Dopamine Release Tracks the Expectation of Dopamine Neuron-Mediated Reinforcement. *Cell Rep. 27*, 481-490.e3.
(147) Gentry, R. N., Lee, B., and Roesch, M. R. (2016) Phasic Dopamine Release in the Rat Nucleus Accumbens Predicts Approach and Avoidance Performance. *Nat. Commun. 7*, 13154.
(148) Howell, J. O., Kuhr, W. G., Ensman, R. E., and Wightman, M. R. (1986) Background Subtraction for Rapid Scan Voltammetry. *J. Electroanal. Chem. 209*, 77–90.
(149) Robinson, D. L., Heien, M. L. A. V, and Wightman, R. M. (2002) Frequency of Dopamine Concentration Transients Increases in Dorsal and Ventral Striatum of Male Rats During Introduction of Conspecifics. *J. Neurosci. 22*, 10477–10486.
(150) Wood, K. M., and Hashemi, P. (2013) Fast-Scan Cyclic Voltammetry Analysis of Dynamic Serotonin Reponses to Acute Escitalopram. *ACS Chem. Neurosci. 4*, 715–720.
(151) Wightman, R. M., and Zimmerman, J. B. (1990) Control of Dopamine Extracellular Concentration in Rat Striatum by Impulse Flow and Uptake. *Brain Res. Rev. 15*, 135–144.
(152) Berglund, E. C., Makos, M. A., Keighron, J. D., Phan, N., Heien, M. L., and Ewing, A. G. (2013) Oral Administration of Methylphenidate Blocks the Effect of Cocaine on Uptake at the *Drosophila* Dopamine Transporter. *ACS Chem. Neurosci. 4*, 566–574.
(153) Makos, M. A., Han, K.-A., Heien, M. L., and Ewing, A. G. (2010) Using *In Vivo* Electrochemistry to Study the Physiological Effects of Cocaine and Other Stimulants on the *Drosophila melanogaster* Dopamine Transporter. *ACS Chem. Neurosci. 1*, 74–83.
(154) Makos, M. A., Kim, Y., Han, K., Heien, M. L., and Ewing, A. G. (2009) *In Vivo*

Electrochemical Measurements of Exogenously Applied Dopamine in *Drosophila melanogaster*. *Anal. Chem. 81*, 1848–1854.
(155) Ciolkowski, E. L., Cooper, B. R., Jankowski, J. A., Jorgenson, J. W., and Wightman, R. M. (1992) Direct Observation of Epinephrine and Norepinephrine Cosecretion from Individual Adrenal Medullary Chromaffin Cells. *J. Am. Chem. Soc. 114*, 2815–2821.
(156) Mosharov, E., and Sulzer, D. (2005) Analysis of Exocytotic Events Recorded by Amperometry. *Nat. Methods 2*, 651–658.
(157) Wightman, R. M., Schroeder, T. J., Finnegan, J. M., Ciolkowski, E. L., and Pihel, K. (1995) Time Course of Release of Catecholamines from Individual Vesicles during Exocytosis at Adrenal Medullary Cells. *Biophys. J. 68*, 383–390.
(158) Amatore, C., Arbault, S., Bonifas, I., Guille, M., Lemaître, F., and Verchier, Y. (2007) Relationship Between Amperometric Pre-Spike Feet and Secretion Granule Composition in Chromaffin Cells: An Overview. *Biophys. Chem. 129*, 181–189.
(159) Oleinick, A., Lemaître, F., Collignon, M. G., Svir, I., and Amatore, C. (2013) Vesicular Release of Neurotransmitters: Converting Amperometric Measurements into Size, Dynamics and Energetics of Initial Fusion Pores. *Faraday Discuss. 164*, 33–55.
(160) Amatore, C., Bouret, Y., Travis, E. R., and Wightman, R. M. (2000) Interplay Between Membrane Dynamics, Diffusion and Swelling Pressure Governs Individual Vesicular Exocytotic Events during Release of Adrenaline by Chromaffin Cells. *Biochimie 82*, 481–496.
(161) Li, Y. T., Zhang, S. H., Wang, L., Xiao, R. R., Liu, W., Zhang, X. W., Zhou, Z., Amatore, C., and Huang, W. H. (2014) Nanoelectrode for Amperometric Monitoring of Individual Vesicular Exocytosis Inside Single Synapses. *Angew. Chemie - Int. Ed. 53*, 12456–12460.
(162) Huang, L., Shen, H., Atkinson, M. A., and Kennedy, R. T. (1995) Detection of Exocytosis at Individual Pancreatic β-Cells by Amperometry at a Chemically Modified Microelectrode. *Proc. Natl. Acad. Sci. U. S. A. 92*, 9608–9612.
(163) Marquis, B. J., McFarland, A. D., Braun, K. L., and Haynes, C. L. (2008) Dynamic Measurement of Altered Chemical Messenger Secretion after Cellular Uptake of Nanoparticles using Carbon-Fiber Microelectrode Amperometry. *Anal. Chem. 80*, 3431–3437.
(164) Majdi, S., Larsson, A., Najafinobar, N., Borges, R., and Ewing, A. G. (2019) Extracellular ATP Regulates the Vesicular Pore Opening in Chromaffin Cells and Increases the Fraction Released During Individual Exocytosis Events. *ACS Chem.*

Neurosci. 10, 2459–2466.
(165) Fisher, R. J., and Burgoyne, R. D. (1999) The Effect of Transfection with Botulinum Neurotoxin C1 Light Chain on Exocytosis Measured in Cell Populations and by Single-Cell Amperometry in PC12 Cells. *Pflugers Arch. Eur. J. Physiol. 437*, 754–762.
(166) Omiatek, D. M., Dong, Y., Heien, M. L., and Ewing, A. G. (2010) Only a Fraction of Quantal Content is Released During Exocytosis as Revealed by Electrochemical Cytometry of Secretory Vesicles. *ACS Chem. Neurosci. 1*, 234–245.
(167) Lovrić, J., Dunevall, J., Larsson, A., Ren, L., Andersson, S., Meibom, A., Malmberg, P., Kurczy, M. E., and Ewing, A. G. (2017) Nano Secondary Ion Mass Spectrometry Imaging of Dopamine Distribution Across Nanometer Vesicles. *ACS Nano 11*, 3446–3455.
(168) Taleat, Z., Larsson, A., and Ewing, A. G. (2019) Anticancer Drug Tamoxifen Affects Catecholamine Transmitter Release and Storage from Single Cells. *ACS Chem. Neurosci. 10*, 2060–2069.
(169) Sakmann, B., and Neher, E. (Eds.). (2009) Single-Channel Recording 2nd edition. Springer Science+Business Media, LLC.
(170) Lindau, M. (2012) High Resolution Electrophysiological Techniques for the Study of Calcium-Activated Exocytosis. *Biochim. Biophys. Acta 1820*, 1234–1242.
(171) Leech, C. A., and Holz, G. G. (1994) Application of Patch Clamp Methods to the Study of Calcium Currents and Calcium Channels. *Methods Cell Biol. 40*, 135–151.
(172) Breckenridge, L. J., and Almers, W. (1987) Currents Through the Fusion Pore that Forms During Exocytosis of a Secretory Vesicle. *Nature 328*, 814–817.
(173) Vangindertael, J., Camacho, R., Sempels, W., Mizuno, H., Dedecker, P., and Janssen, K. P. F. (2018) An Introduction to Optical Super-Resolution Microscopy for the Adventurous Biologist. *Methods Appl. Fluoresc. 6*.
(174) Ingle, J. D., and Crouch, S. R. (1988) Spectrochemical Analysis. Prentice-Hall, Inc.
(175) Gubernator, N. G., Zhang, H., Staal, R. G. W. W., Mosharov, E. V., Pereira, D. B., Yue, M., Balsanek, V., Vadola, P. A., Mukherjee, B., Edwards, R. H., Sulzer, D., and Sames, D. (2009) Fluorescent False Neurotransmitters Visualize Dopamine Release from Individual Presynaptic Terminals. *Science (80-.). 324*, 1441–1444.
(176) Shimomura, O., Johnson, F. H., and Saiga, Y. (1962) Extraction, Purification, and Properties of Aequorin, a Bioluminescent Protein from the Luminus Hydromedusan, Aequorea. *J. Cell. Comp. Biol. 59*, 223–239.
(177) Cranfill, P. J., Sell, B. R., Baird, M. A., Allen, J. R., Lavagnino, Z., De Gruiter, H.

M., Kremers, G. J., Davidson, M. W., Ustione, A., and Piston, D. W. (2016) Quantitative Assessment of Fluorescent Proteins. *Nat. Methods 13*, 557–562.
(178) Tsien, R. Y. (1998) The Green Fluorescent Protein. *Annu. Rev. Biochem. 67*, 509–544.
(179) Lang, T., Wacker, I., Steyer, J., Kaether, C., Wunderlich, I., Soldati, T., Gerdes, H. H., and Almers, W. (1997) Ca^{2+}-Triggered Peptide Secretion in Single Cells Imaged with Green Fluorescent Protein and Evanescent-Wave Microscopy. *Neuron 18*, 857–863.
(180) Crivat, G., and Taraska, J. W. (2012) Imaging Proteins Inside Cells with Fluorescent Tags. *Trends Biotechnol. 30*, 8–16.
(181) Janeway, C. A., Travers, P. J., Walport, M., and Shlomchik, M. J. (2001) Immunobiology: The Immune System in Health and Disease 5th edition. Garland Science, New York.
(182) Maidorn, M., Rizzoli, S. O., and Opazo, F. (2016) Tools and Limitations to Study the Molecular Composition of Synapses by Fluorescence Microscopy. *Biochem. J. 473*, 3385–3399.
(183) Grynkiewicz, G., Poenie, M., and Tsien, R. Y. (1985) A New Generation of Ca^{2+} Indicators with Greatly Improved Fluorescence Properties. *J. Biol. Chem. 260*, 3440–3450.
(184) Ormö, M., Cubitt, A. B., Kallio, K., Gross, L. A., Tsien, R. Y., and Remington, S. J. (1996) Crystal Structure of the *Aequorea victoria* Green Fluorescent Protein. *Science (80-.). 273*, 1392–1395.
(185) Tran, D. T., Masedunskas, A., Weigert, R., and Ten Hagen, K. G. (2015) Arp2/3-Mediated F-actin Formation Controls Regulated Exocytosis In Vivo. *Nat. Commun. 6*, 10098.
(186) Harke, B., Keller, J., Ullal, C. K., Westphal, V., Schönle, A., and Hell, S. W. (2008) Resolution Scaling in STED Microscopy. *Opt. Express 16*, 4154–4162.
(187) Xu, K., Babcock, H. P., and Zhuang, X. (2012) Dual-Objective STORM Reveals Three-Dimensional Filament Organization in the Actin Cytoskeleton. *Nat. Methods 9*, 185–188.
(188) Rust, M. J., Bates, M., and Zhuang, X. (2010) Sub-Diffraction-Limit Imaging with Stochastic Optical Reconstruction Microscopy (STORM). *Nat. Methods 3*, 793–795.
(189) Willig, K. I., Rizzoli, S. O., Westphal, V., Jahn, R., and Hell, S. W. (2006) STED Microscopy Reveals that Synaptotagmin Remains Clustered after Synaptic Vesicle Exocytosis. *Nature 440*, 935–939.

(190) Rahbek-Clemmensen, T., Lycas, M. D., Erlendsson, S., Eriksen, J., Apuschkin, M., Vilhardt, F., Jørgensen, T. N., Hansen, F. H., and Gether, U. (2017) Super-Resolution Microscopy Reveals Functional Organization of Dopamine Transporters into Cholesterol and Neuronal Activity-Dependent Nanodomains. *Nat. Commun. 8*, 740.
(191) JEOL TEM Terms (https://www.jeol.co.jp/en/words/emterms/search_result.html?keyword=wavelength%20of%20electron).
(192) Boekema, E. J., Folea, M., and Kouril, R. (2009) Single Particle Electron Microscopy. *Photosynth. Res. 102*, 189–196.
(193) Conley, M. J., and Bhella, D. (2019) Asymmetric Analysis Reveals Novel Virus Capsid Features. *Biophys. Rev. 11*, 603–609.
(194) Bozzola, J. J., and Russell, L. D. (1992) Electron Microscopy 1st edition. Jones and Bartlett Publishers, Inc.
(195) Hayat, M. A. (2000) Electron Microscopy: Biological Applications 4th edition. Cambridge University Press.
(196) Steinbrecht, R. A., and Zierold, K. (1987) Cryotechniques in Biological Electron Microscopy 1st edition. Springer-Verlag, Berlin.
(197) Dubochet, J., and McDowall, A. W. (1981) Vitrification of Pure Water for Electron Microscopy. *J. Microsc. 124*, RP3–RP4.
(198) Unwin, P. N. T., and Henderson, R. (1975) Molecular Structure Determination by Electron Microscopy of Unstained Crystalline Specimens. *J. Mol. Biol. 94*, 425–440.
(199) McIntosh, R., Nicastro, D., and Mastronarde, D. (2005) New Views of Cells in 3D: An Introduction to Electron Tomography. *Trends Cell Biol. 15*, 43–51.
(200) Perkins, G. A., Jackson, D. R., and Spirou, G. A. (2015) Resolving Presynaptic Structure by Electron Tomography. *Synapse 69*, 268–282.
(201) Frank, J. (2005) Electron Tomography: Methods for Three-Dimensional Visualization of Structures in the Cell 2nd edition. Springer.
(202) Chandler, D. E., and Heuser, J. E. (1980) Arrest of Membrane Fusion Events in Mast Cells by Quick-Freezing. *J. Cell Biol. 86*, 666–674.
(203) Nakata, T., Sobue, K., and Hirokawa, N. (1990) Conformational Change and Localization of Calpactin I Complex Involved in Exocytosis. *J. Cell Biol. 110*, 13–25.
(204) Savigny, P., Evans, J., and McGrath, K. M. (2007) Cell Membrane Structures during Exocytosis. *Endocrinology 148*, 3863–3874.
(205) Araç, D., Chen, X., Khant, H. A., Ubach, J., Ludtke, S. J., Kikkawa, M., Johnson, A. E., Chiu, W., Südhof, T. C., and Rizo, J. (2006) Close Membrane-Membrane

Proximity Induced by Ca^{2+}-Dependent Multivalent Binding of Synaptotagmin-1 to Phospholipids. *Nat. Struct. Mol. Biol. 13*, 209–217.
(206) De Boer, P., Hoogenboom, J. P., and Giepmans, B. N. G. (2015) Correlated Light and Electron Microscopy: Ultrastructure Lights Up ! *Nat. Methods 12*, 503–513.
(207) Müller, A., Neukam, M., Ivanova, A., Sönmez, A., Münster, C., Kretschmar, S., Kalaidzidis, Y., Kurth, T., Verbavatz, J. M., and Solimena, M. (2017) A Global Approach for Quantitative Super Resolution and Electron Microscopy on Cryo and Epoxy Sections Using Self-labeling Protein Tags. *Sci. Rep. 7*, 23.
(208) Begemann, I., and Galic, M. (2016) Correlative Light Electron Microscopy: Connecting Synaptic Structure and Function. *Front. Synaptic Neurosci. 8*, 28.
(209) Hoffmann, E. De, and Stroobant, V. (2007) Mass Spectrometry Principles and Applications 3rd edition. John Wiley & Sons, Ltd.
(210) Karas, M., Bachmann, D., and Hillenkamp, F. (1985) Influence of the Wavelength in High-Irradiance Ultraviolet Laser Desorption Mass Spectrometry of Organic Molecules. *Anal. Chem. 57*, 2935–2939.
(211) Tanaka, K., Waki, H., Ido, Y., Akita, S., and Yoshida, Y. (1988) Protein and Polymer Analyses up to m/z 100 000 by Laser Ionization Time-of-flight Mass Spectrometry. *Rapid Commun. Mass Spectrom. 2*, 151–153.
(212) Vickerman, J. C. (2011) Molecular Imaging and Depth Profiling by Mass Spectrometry - SIMS, MALDI or DESI ? *Analyst 136*, 2199–2217.
(213) MacAleese, L., Stauber, J., and Heeren, R. M. A. (2009) Perspectives for Imaging Mass Spectrometry in the Proteomics Landscape. *Proteomics 9*, 819–834.
(214) Hanreider, J., Phan, N. N. T., Kurczy, M. E., and Ewing, A. G. (2013) Imaging Mass Spectrometry in Neuroscience. *ACS Chem. Neurosci. 4*, 666–679.
(215) Cameca NanoSIMS 50L (https://www.cameca.com/products/sims/nanosims).
(216) Benabdellah, F., Seyer, A., Quinton, L., Touboul, D., Brunelle, A., and Laprévote, O. (2010) Mass Spectrometry Imaging of Rat Brain Sections: Nanomolar Sensitivity with MALDI Versus Nanometer Resolution by TOF-SIMS. *Anal. Bioanal. Chem. 396*, 151–162.
(217) Buchberger, A. R., DeLaney, K., Johnson, J., and Li, L. (2018) Mass Spectrometry Imaging: A Review of Emerging Advancements and Future Insights. *Anal. Chem. 90*, 240–265.
(218) Tian, H., Sparveto, L. J., Amoscato, A. A., Bloom, A., Bayir, H., Kagan, V. E., and Winograd, N. (2017) Gas Cluster Ion Beam Time-of-Flight Secondary Ion Mass Spectrometry High-Resolution Imaging of Cardiolipin Speciation in the Brain: Identification of Molecular Losses after Trumatic Injury. *Anal. Chem. 89*, 4611–4619.

(219) Tatsuta, Y., Kasai, K., Maruyama, C., Hamano, Y., Matsuo, K., Katano, H., and Taira, S. (2017) Imaging Mass Spectrometry Analysis of Ubiquinol Localization in the Mouse Brain Following Short-Term Administration. *Sci. Rep. 7*, 12990.
(220) Lanekoff, I., Kurczy, M. E., Hill, R., Fletcher, J. S., Vickerman, J. C., Winograd, N., Sjövall, P., and Ewing, A. G. (2010) Time of Flight Mass Spectrometry Imaging of Samples Fractured *In Situ* with a Spring-Loaded Trap System. *Anal. Chem. 82*, 6652–6659.
(221) Zimmerman, T. A., Rubakhin, S. S., and Sweedler, J. V. (2011) MALDI Mass Spectrometry Imaging of Neuronal Cell Cultures. *J. Am. Soc. Mass Spectrom. 22*, 828–836.
(222) Ren, L., Dowlatshahi Pour, M., Malmberg, P., and Ewing, A. G. (2019) Altered Lipid Composition of Secretory Cells Following Exposure to Zinc Can Be Correlated to Changes in Exocytosis. *Chem. - Eur. J. 25*, 5406–5411.
(223) Mohammadi, A. S., Li, X., and Ewing, A. G. (2018) Mass Spectrometry Imaging Suggests That Cisplatin Affects Exocytotic Release by Alteration of Cell Membrane Lipids. *Anal. Chem. 90*, 8509–8516.
(224) Saka, S. K., Vogts, A., Kröhnert, K., Hillion, F., Rizzoli, S. O., and Wessels, J. T. (2014) Correlated Optical and Isotopic Nanoscopy. *Nat. Commun. 5*, 3664.