Thesis for the Degree of Doctor of Philosophy

Analytical Approaches to Study Vesicular and Exosomal Release

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UNIVERSITY OF GOTHENBURG

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Abstract

Vesicles, as a special type of entity nanometers in size, are crucial for survival of multicellular organisms. Intracellular vesicles mainly contain specific signaling molecules, transmitters, and modulators whereas extracellular vesicles (EVs) are bioactive organelles carrying a wide range of proteins, genetic materials, and other molecules. Secretion from vesicles is essential to manipulate many biological pathways and intercellular communication. Understanding the regulatory mechanisms of vesicular secretion is mandatory for uncovering the pathologies of neurological disorders and developing related pharmaceuticals.

Several electrochemical techniques have been proposed and developed to unravel vesicular neurotransmitters at single cell and subcellular levels. These methodologies provide high spatiotemporal resolution and sensitivity while enabling direct quantification of electroactive molecules from individual vesicles. Single cell amperometry (SCA) can be employed to determine the number of signaling transmitters being released during an exocytosis event. Vesicle impact electrochemical cytometry (VIEC) and intracellular vesicle impact electrochemical cytometry (IVIEC) are two methods that allow the quantification of the number of signaling molecules stored inside single vesicles from isolated or intracellular vesicles, respectively.

In this thesis work, vesicular structure as well as their content and release have been investigated. In paper I, open carbon nanopipettes (CNPs) with different radii between 50 and 600 nm were employed to quantify vesicular content in isolated vesicles of adrenal chromaffin cells by VIEC. Paper II was continuation of work from paper I, the mechanistic study of L-DOPA was carried out by using IVIEC with various sized CNPs. SCA was introduced to capture the dynamic release of single exosomes from a single living cell in paper III. In paper IV, the combination of electrochemistry and mass spectrometry was applied to investigate the effects of ketamine on dopamine storage and exocytosis, as well as the alterations of cellular lipid composition. Generally, electrochemical methods have been considered as a powerful tool to understand structures of vesicles and their biological functions.

Sammanfattning på svenska

Vesiklar, som är en speciell typ av nanometerstora enheter, är avgörande för multicellulära organismers överlevnad. Intracellulära vesiklar innehåller huvudsakligen specifika signalmolekyler, transmittorer och modulatorer, medan extracellulära vesiklar (EVs) är bioaktiva organeller som innehåller ett brett spektrum av proteiner, genetiska material och andra molekyler. Utsöndring från vesiklar är avgörande för att manipulera många biologiska vägar och intercellulär kommunikation. Att förstå reglermekanismerna för vesikulär utsöndring är nödvändigt för att avslöja patologin bakom neurologiska sjukdomar och utveckla relaterade läkemedel.

Flera elektrokemiska tekniker har föreslagits och utvecklats för att kartlägga vesikulära neurotransmittorer på cell och subcellulär nivå. Dessa metoder ger hög spatiotemporal upplösning och känslighet samtidigt som de möjliggör direkt kvantifiering av elektroaktiva molekyler från enskilda vesiklar. Single cell amperometry (SCA) kan användas för att bestämma antalet signalsubstanser som frisätts under en exocytos. Vesicle impact electrochemical cytometry (VIEC) och intracellular vesicle impact electrochemical cytometry (IVIEC) är två metoder som möjliggör kvantifiering av antalet signalmolekyler som lagras i enskilda vesiklar från isolerade respektive intracellulära vesiklar.

I detta avhandlingsarbete har vesikulär struktur samt deras innehåll och frisättning undersökts. I artikel I användes öppna carbon nanopipettes (CNPs) med olika radier mellan 50 och 600 nm för att kvantifiera vesikulärt innehåll i isolerade vesiklar från kromaffin celler med hjälp av VIEC. Artikel II var en fortsättning på arbetet från artikel I, den mekanistiska studien av L-DOPA utfördes med hjälp av IVIEC med CNPs i olika storlekar. SCA introducerades för att fånga den dynamiska frisättningen av enstaka exosomer från en enda levande cell i artikel III. I artikel IV användes en kombination av elektrokemi och masspektrometri för att undersöka effekterna av ketamin på dopaminlagring och exocytos, samt förändringarna av cellulär lipidsammansättning. Generellt har elektrokemiska metoder ansetts vara kraftfulla verktyg för att förstå strukturer hos vesiklar och deras biologiska funktioner.

List of Publications and Contribution Report

I. Correlating Molecule Count and Release Kinetics with Vesicular Size Using Open Carbon Nanopipettes

Keke Hu, Rui Jia, Amir Hatamie, Kim Long Le Vo, Michael V. Mirkin, and Andrew G. Ewing

Journal of the American Chemical Society 2020, 142, 16910–16914.

Performed the electrochemical experiments and participated in data interpretation. Edited the manuscript with the other authors.

II. Quantifying Intracellular Single Vesicular Catecholamine Concentration with Open Carbon Nanopipettes to Unveil the Effect of L-DOPA on Vesicular Structure

Kim Long Le Vo[†], Keke Hu[†], Amir Hatamie, and Andrew G. Ewing

Angewandte Chemie International Edition 2022, 61, e20211340

Performed the electrochemical experiments, analyzed and interpreted the data. Participated in writing and editing the manuscript with Keke Hu.

III. Single Exosomes Amperometric Measurements Reveal Encapsulation of Chemical Messengers for Intercellular Communication

Kim Long Le Vo[†], Keke Hu[†], Fan Wang, Xin Zhang, Chaoyi Gu, Ning Fang, Nhu T. N. Phan, and Andrew G. Ewing

Journal of the American Chemical Society 2023, 145, 11499–11503

Contributed to tailor the project. Performed the electrochemical experiments, analyzed and interpreted the data. Participated in data interpretation with other co-authors. Wrote and edited the manuscript with Keke Hu.

IV. Electrochemical and Mass Spectrometric Measurement of Enhanced Intravesicular Catecholamine Content and Exocytotic Frequency at Subanaesthetic Ketamine Doses

Zhaoying Wang[†], Xiulan He[†], Kim Long Le Vo, and Andrew G. Ewing

Analysis & Sensing 2021, 1, 166–170.

Participated in sample preparation and performed electrochemical experiments with Xiulan He. Analyzed some of the data and edited the manuscript with others.

[†] *These authors contributed equally to the work.*

Related Publications not included in the thesis

Chemical Imaging and Analysis of Single Nerve Cells Using Secondary Ion Mass Spectrometry Imaging and Cellular Electrochemistry

Alicia Andrea Lork, Kim Long Le Vo, and Nhu T. N. Phan
Frontiers in Synaptic Neuroscience 2022, 14, 854957

Understanding the physiological functions of stress granules in neurotransmission

Keke Hu, Kim Long Le Vo, Alicia Andrea Lork, Andre Du Toit, Nhu T. N. Phan, and Andrew G. Ewing
Manuscript in preparation

Effects of Alpha Synuclein on Exocytosis and Efficacy of Amantadine in Rescuing Vesicular Storage

Kim Long Le Vo, Stefania Rabasco, Tho Duc Khanh Nguyen, Ranjeet Kumar, Samantha Soroori, Pernilla Wittung-Stafshede, and Andrew G. Ewing
Manuscript in preparation

Regulation of Exocytosis During Abeta Amyloid Aggregation

Kim Long Le Vo, Tho Duc Khanh Nguyen, Xiulan He, Lin Ren, David Bernson, Nima Sasanian, Elin K. Esbjörner, and Andrew G. Ewing
Manuscript in preparation

Table of contents

ABBREVIATIONS

ADP – Adenosine diphosphate

ATP – Adenosine triphosphate

CNPs – Carbon nanopipettes

CNS – Central nervous system

COMT – Catechol-O-methyl transferase

ESCRT – Endosomal sorting complex required for transport

EVs – Extracellular vesicles

IVIEC – Intracellular vesicle impact electrochemical cytometry

LDCVs – Large dense core vesicles

L-DOPA – L-3,4-dihydroxyphenylalanine

MAO – Monoamine oxidase

NDMA – N-methyl-D-aspartate

NSF – N-ethylmaleimide-sensitive-factor

PC12 – Pheochromocytoma

PNMT – Phenylethanolamine-N-methyltransferase

PNS – Peripheral nervous system

V-ATPase – Vacuolar-type H^+-ATPase

VIEC – Vesicle impact electrochemical cytometry

VMAT – Vesicular monoamine transporter

VNUT – Vesicular nucleotide transporter

t-SNAREs – Target SNAREs

v-SNARE – Vesicle SNARE

SCA – Single cell amperometry

SIMS – Secondary ion mass spectrometry

SNARE – Soluble N-ethylmaleimide-sensitive-factor attachment protein receptor

SSVs – Small synaptic vesicles

TEM – Transmission electron microscopy

ToF-SIMS – Time-of-flight SIMS

CHAPTER 1. Introduction to cellular communication

Cellular communication or cell signalling is a critical process for cell growth, development, differentiation, migration, and apoptosis. In multicellular organisms, cell to cell communication is vital for the transmission of biological information to survive and flourish. Malfunctions in cellular communication can cause abnormal regulation of the cell cycle, which leads to the formation of various diseases including cancer, neurodegenerative disorders, and cognitive deficiency. This chapter introduces an overview of the anatomy and functions of the neuronal cells and nervous system. Moreover, different types of neurotransmitters and their biosynthetic pathway are also included.

1.1 An overview of nervous system

1.1.1 The neuronal cells

The human brain is comprised of a billion nerve cells known as neurons. These neurons are responsible for processing and transmitting information from the brain to other parts of the body. Although neurons are different from other cell types and can be classified into various categories depending on their morphologies and functions, they all include a cell body, dendrites, and an axon (Figure 1). The cell body, also called the soma, contains the nucleus and major cytoplasmic organelles that can be found in other cells, such as Golgi apparatus, mitochondria, ribosomes, and endoplasmic reticulum. It stores all genetic information and directs protein synthesis to support and maintain neuronal function. Dendrites, a branch-like structure, are fibrous roots extended from the cell body. Generally, their functions are like antennas, they can receive and process synaptic signal inputs from other neurons. The axon, a long tail-like structure, plays an important role in transferring signals away from the soma to other neurons. For many neurons, a fatty substance coated around the nerve axon is called myelin that acts as an insulator. Myelin sheaths help to promote the

propagation of action potentials along the axon and speed up the transmitting information process between neurons.

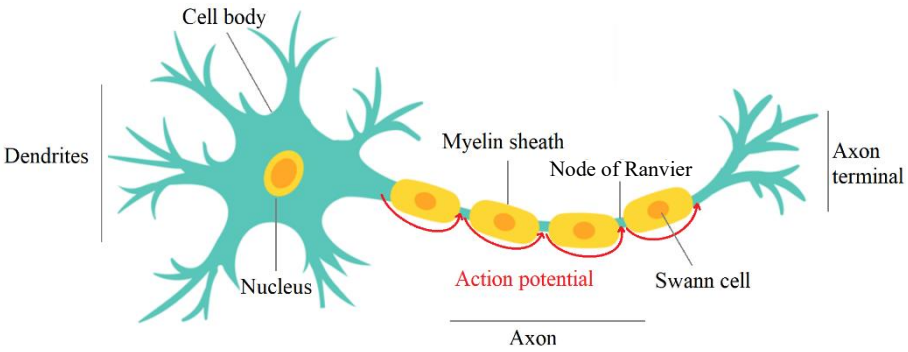

Figure 1. An illustration of a nerve cell. A typical neuron structure consists of dendrites, cell body (soma), axon, and axon terminal. An action potential is propagated along the axon by jumping between unmyelinated gaps called nodes of Ranvier.

1.1.2 The nervous system

The nervous system consists of two major components, the central nervous system (CNS) and the peripheral nervous system (PNS), where the CNS as the control center is made up of the brain and the spinal cord, and the PNS, composed of branching nerves originating from the brain and the spinal cord, contains the sensory neurons and the motor neurons. The function of the sensory neurons, or afferent neurons, is to carry the information from sensory receptors, which are located either at the surface of the body such as skin or deeper into other internal organs or tissues, towards the CNS. The motor neurons, also known as efferent neurons, are additionally classified into the somatic nervous system, regulating voluntary movements via skeletal muscles, and autonomic nervous system, associated with the innervation of involuntary activities coming from the visceral organs (like smooth muscles, cardiac muscles, and glands) concerning blood pressure, heartbeat, breathing rate, etc. The autonomic system is further subdivided into two branches, one is the sympathetic system, and the other is the parasympathetic system. The sympathetic system is

commonly known as the 'fight or flight' system while the parasympathetic system is often considered the 'rest and digest' system. In other words, these antagonistic functions are the keys to balancing homeostasis in the body. The fundamental units of the nervous system are neurons, involved in receiving and transmitting chemical or electrical signals, and glia cells which play supportive and protective roles for neurons by modulating their function and signaling. For example, glia can manipulate synapse formation, regulate blood flow and metabolism, maintain ionic and water homeostasis for signal transduction, and form myelin sheath from the Schwann cells for insulating axons.

1.2 Cellular communication within the nervous system

Neurons are responsible for cellular communication in the nervous system and one neuron can send a message to another one via a junction called synapse. The sending process is known as synaptic transmission and the neuron that generates an action potential is called a presynaptic cell while the target neuron receiving an action potential is called a postsynaptic cell. Action potentials are fundamental units that help neurons to communicate with each other by rapidly alternating the voltage across nerve cell membrane.

In neurons, the typical membrane potential maintains between -50 and -75mV at the resting state. It depends on the cell type and the difference of ionic concentrations between the intracellular and extracellular environments. This value is defined by an uneven distribution of different ions across the membrane and termed the resting membrane potential.[1,2] Generally, K^+ ions are basically found at higher concentrations within the cell than outside, whereas Na^+ and Cl^- ion concentrations are higher in the extracellular space.[3] There are also organic anions, found in proteins and amino acids, inside the cell and these negatively charged molecules cannot travel freely through the hydrophobic regions of the membrane. The resting membrane is more permeable towards K^+ than the other ions in the nerve cells, so the resting potential is nearest to the equilibrium potential of K^+ ions and the voltage is around -75 mV. The Na^+/K^+ pump, an ATPase, helps to

establish the concentration gradients via active transport by pumping K^+ ions into the cell and excess Na^+ ions out of the cell, which maintains the membrane potential.

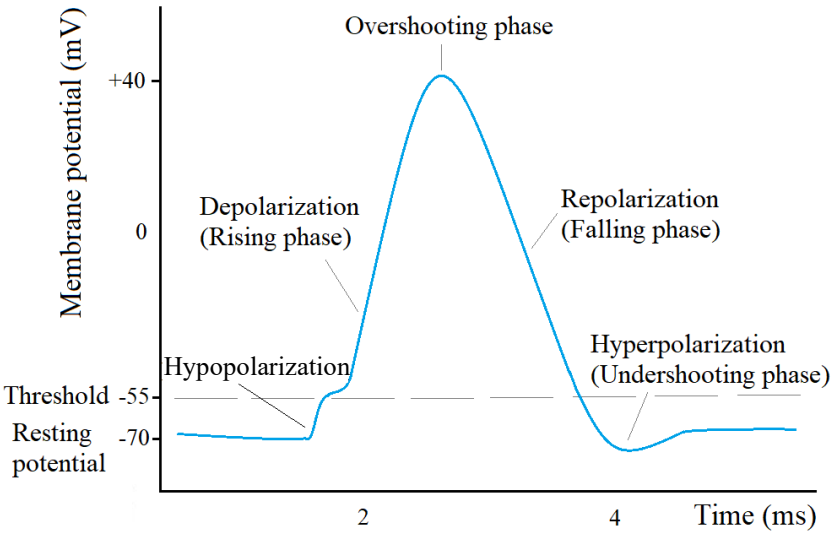

Figure 2. Diagram showing different phases of an action potential in relation to the membrane voltage over time.

An action potential is successfully generated if a stimulus can increase the membrane potential to reach a certain threshold, usually around -50 to -55 mV. Moreover, action potentials are described as 'all or none', as subthreshold stimuli will not give any changes while threshold or suprathreshold stimuli can trigger an action potential at the axon hillock. All different stages of an action potential are shown in Figure 2. The initial increase from the resting potential to the threshold potential is called hypopolarization. At the potential of -55 mV, the voltage-gated Na^+ channels are activated and cause a large influx of Na^+ ions during the depolarization process or rising phase. The membrane potential becomes more positive until reaching the equilibrium potential of Na^+, around +55 mV, as the overshooting phase. After that, the voltage-gated Na^+ channels begin to close and the voltage-gated K^+ channels are open, which enables K^+ ions to leak out of the cell. The

membrane potential is decreased and becomes more negative in the falling phase or the repolarization due to the migration of positive K^+ ions to the extracellular fluid. This progress will not stop until the voltage is lower than the default resting potential, shown as the undershooting phase or the hyperpolarization. But soon after that, the voltage-gated K^+ channels are deactivated to bring back the potential to the resting state and the Na^+/K^+ ATPase pumps are activated to balance the membrane potential.

To keep the same amplitude of an action potential along the axon, the process described above needs to be constantly replicated from the beginning of the axon toward the axon terminal. In unmyelinated axons, action potentials are propagated as continuous conduction, which requires activation of voltage-gated Na^+ channels along the entire length of the membrane. On the contrary, propagation along a myelinated axon, containing fatty mostly lipid coating to insulate the nerve cells, is referred as saltatory conduction because the action potential needs to jump between unmyelinated gaps known as nodes of Ranvier (Figure 1), where the voltage-gated Na^+ channels are clustered. The speed of action potential propagation is particularly critical for information communication in neurons, and it also depends on the thickness of the axon, especially for unmyelinated axons.

Synaptic transmission is a vital process for nerve cells to communicate and is driven by the action potential. There are two classes of synaptic transmission: electrical and chemical. In an electrical synapse, the membranes of two neurons are very close to each other and physically connected by a protein channel forming a gap junction (about 4 nm diameter) which directly allows ion currents to flow from one neuron to the next. Therefore, signaling in electrical synapses is virtually instantaneous and the transmission is usually bidirectional, which means that current can diffuse in either direction across the gap junction. Electrical synapses play an important role for synchronizing the electrical activity in a group of neurons.

However, chemical synaptic transmission is more common and complicated in cellular signaling. This process involves the release of

neurotransmitter molecules as chemical messengers in vesicles, carrying information from the presynaptic neuron to the postsynaptic neuron. To preserve neuronal communication, close contact between the two nerve cells, or synaptic cleft, from 20 to 40 nm, needs to be maintained.[4] When an action potential arrives at the axon terminal, it induces the depolarization of the plasma membrane and activates voltage-gated calcium channels in the membrane. As the concentration of Ca^{2+} ions is higher outside the neuron than inside, Ca^{2+} ions enter the cell starting off a signaling cascade that allows secretory vesicles to fuse with the axon terminal membrane. This process causes neurotransmitter release into the synaptic cleft, also known as exocytosis, and is described in more detail in the next chapter. Diffusion of transmitter molecules across the synaptic cleft leads to the binding between neurotransmitters and specific receptors on the plasma membrane of the receiving neurons. Depending on the properties of transmitters, the postsynaptic membrane potential is either increased, an excitation effect to fire an action potential, or decreased, an inhibitory effect which reduces generation of an action potential. Outside the general model, release of transmitters is also found at extra synaptic regions including the cell soma and the dendrites.[5] After synaptic transmission is completed, neurotransmitters must be removed by different mechanisms so that the postsynaptic membrane can receive another signal. For instance, transmitter molecules can be degraded by enzymes, or recycled by active transport through a transporter or reuptake pump.[6]

1.3 Different types of transmitters

In order for neurons to communicate with each other, chemical messengers called neurotransmitters need to be released in the synaptic cleft. More than 100 distinct chemical messengers have been identified up to date. Neurotransmitters, at the highest level, can be categorized into two broad groups simply based on their sizes: small-molecule transmitters and neuropeptides. Small-molecule neurotransmitters are synthesized in the axonal terminal and are generally stored in small synaptic vesicles while neuropeptides require more complicated

synthesis with the formation of peptide bond and are accumulated in large dense core vesicles. Depending on their chemical structures, small-molecule transmitters can be grouped into four different classes: acetylcholine, amino acids (glutamic acid, aspartic acid, γ-aminobutyric acid, and glycine), purines, and biogenic amines (dopamine, epinephrine, norepinephrine, serotonin, and histamine). In this thesis work, the three catecholamines including dopamine, epinephrine and norepinephrine are mainly investigated. A schematic diagram of the biosynthetic pathway for dopamine, epinephrine and norepinephrine is shown in Figure 3.

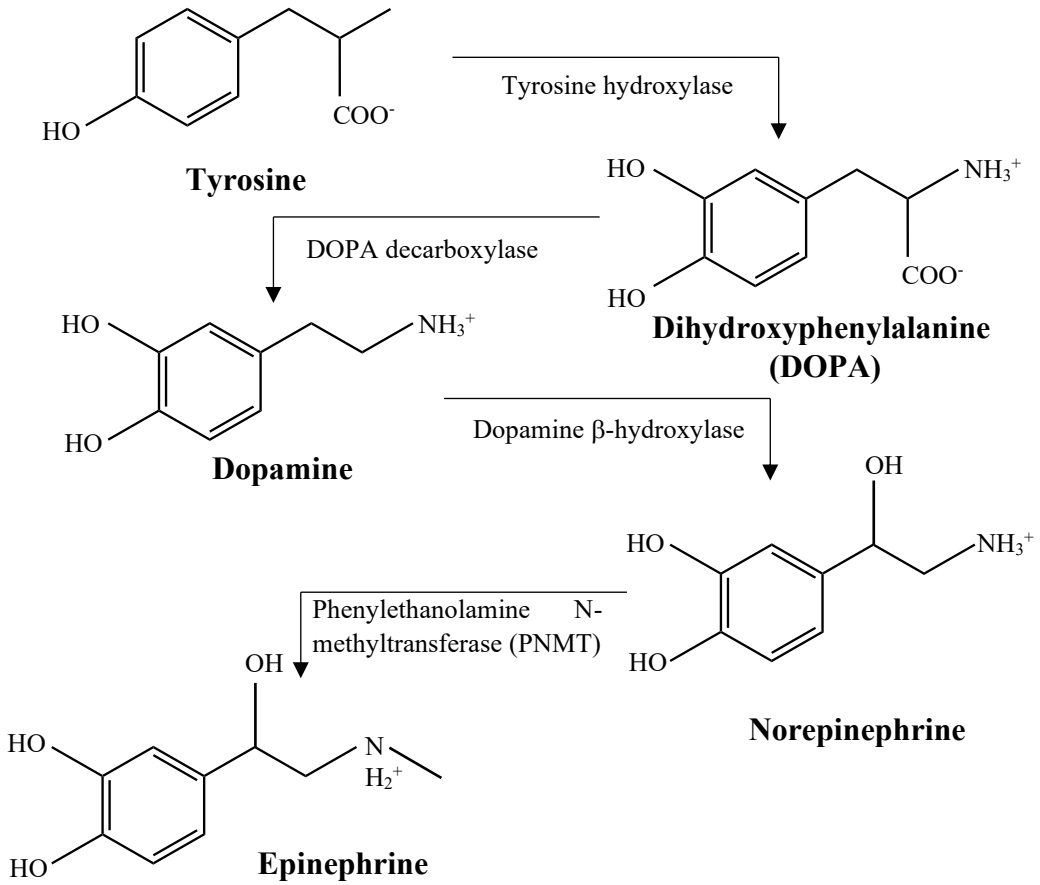

Figure 3. Chemical structures of three catecholamines and their biosynthetic pathway.

1.3.1 Dopamine

Dopamine was discovered as a neurotransmitter in the brain by Arvid Carlsson more than 50 years ago.[7] Subsequently, Paul Greengard provided the cellular signalling mechanisms of dopamine, and these two pioneers were awarded the Nobel Prize for Physiology and Medicine in 2000. As one of the major neurotransmitters in the brain, dopamine is crucial for movement regulation,[8] motivation,[9] and learning and memory.[10] Dysfunction of dopaminergic systems leads to different neurodegenerative disorders such as Parkinson's disease, schizophrenia, or attention deficit hyperactivity disorder.

Dopamine is synthesized either directly from tyrosine or indirectly from phenylalanine, which can be converted to tyrosine by phenylalanine hydroxylase.[11,12] Regardless, the primary metabolic route of dopamine containing two steps takes place in the cytosol as dopamine is unable to cross the blood-brain barrier. The classical biosynthesis pathway of dopamine was reported by Blaschko in 1939.[13] Firstly, tyrosine is converted to L-3,4-dihydroxyphenylalanine (L-DOPA) by the cytosolic enzyme tyrosine hydroxylase together with the presence of tetrahydrobiopterin, oxygen (O_2), and iron (Fe^{2+}) as cofactors. DOPA decarboxylase is then responsible for the conversion of L-DOPA to dopamine, using pyridoxal phosphate as a cofactor.[14] After synthesis, the newly dopamine molecules are transferred into the acidic lumen of synaptic vesicles via the vesicular monoamine transporter (VMAT).[15] These vesicles are stored in the presynaptic terminal and dopamine is mainly released through a process called exocytosis, which leads to a change in receptors at the pre- and postsynaptic membranes.[15–17] In this way, dopamine can bind to or activate both presynaptic and postsynaptic receptors to modulate cyclic adenosine monophosphate generation. There are five subtypes of dopamine G-protein-coupled receptors, D1-D5, which are classified into two families, D1-like receptor including D1 and D5 receptors, and D2-like receptor involving D2, D3 and D4 receptors.[18] After conducting its synaptic function, extracellular dopamine needs to be cleaned from the cleft to terminate signaling. It can be taken up in secretory vesicles in the cytoplasm by the action of the dopamine transporter or monoamine transporter.[8,19]

Dopamine degradation is effectively mediated by monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT) into inactive metabolites 3,4-dihydroxyphenylacetic acid and homovanillic acid.

1.3.2 Epinephrine and norepinephrine

Epinephrine, also called adrenaline, is normally produced by the adrenal medulla or present only in epinephrine-containing neurons in the CNS at lower levels than the other two catecholamines. Norepinephrine, known as noradrenaline, is predominantly generated through locus coeruleus noradrenergic neurons in the brain or sympathetic nerve fibers in the body, and is less abundant in adrenal glands. Epinephrine release can cause contractions to or relaxations for various organs while the level of norepinephrine in the brain is related to sleep and wakefulness.[20] Together, they play an essential role to induce the fight-or-flight response under stressful or dangerous situations.[21]

Norepinephrine is synthesized from dopamine inside synaptic vesicles. Once dopamine is packaged in the vesicles, the conversion of norepinephrine is catalyzed by a membrane bound enzyme called dopamine-β-hydroxylase with the presence of oxygen and ascorbic acid. In contrast, phenylethanolamine-N-methyltransferase (PNMT), the main enzyme responsible for the formation of epinephrine from norepinephrine, is located in the cytosol of the cell. Therefore, vesicular norepinephrine needs to leave the vesicles into the cytoplasm prior to synthesis of epinephrine. Norepinephrine is then converted into epinephrine by the enzyme PNMT and S-adenosylmethionine as a cofactor.[22] Subsequently, the synthesized epinephrine molecules are repackaged into the vesicles via the VMAT. Upon being secreted, these two catecholamines can bind to membrane G-protein coupled receptors called adrenergic receptors, which include α- and β-adrenergic receptors. They can interact with both types of receptors; however, norepinephrine preferably binds to α-adrenergic receptors whereas epinephrine has stronger affinity for β-adrenergic receptors. The majority of released epinephrine and norepinephrine can be cleared from synapses via the reuptake mechanism. This process is mediated by

the norepinephrine transporter and the neurotransmitters are taken up into the presynaptic cell. In addition, epinephrine and norepinephrine are metabolized to 3-methoxy-4-hydroxy-phenylglycol by two enzymes MAO and COMT, the same enzymes for breaking down dopamine.

CHAPTER 2. Vesicles and their secretory pathways

Intercellular communication is initiated by the generation and release of specific vesicles with membrane-bound structures. This process is mediated by the regulated fusion of secretory vesicles with the cell membrane leading to release neurotransmitters, hormones, neuropeptides, and proteins, known as exocytosis, or by constitutive secretion of extracellular vesicles (EVs) to transport proteins, enzymes, nucleic acids, and lipids. Dysfunction of vesicle trafficking can cause the development of various diseases and disorders. In this chapter, the fundamental information of different types of vesicles and their structures is discussed. According to distinct properties of specific vesicles, the vesicular trafficking and secretion pathways are also summarized including conventional and unconventional processes.

2.1 Secretory vesicles

Vesicles are small and spherical compartments derived from the Golgi apparatus. Those organelles that can store and release neurotransmitters are known as secretory vesicles. Basically, they are categorized into two main groups, small synaptic vesicles (SSVs) and large dense core vesicles (LDCVs), depending on their morphology, distribution, and release kinetics.[23–27] Both types of vesicles are responsible for different functions in the nervous system.[26,28] SSVs have a diameter of 40-60 nm and contain classical neurotransmitters such as catecholamines, acetylcholine, glutamate, or γ-amino butyric acid. The centers of these vesicles appear to be electron-lucent and clear in transmission electron microscopy (TEM). In contrast, LDCVs are bigger, the diameter between 100-300 nm, and carry neurotransmitters such as biogenic amines, catecholamines, neuropeptides and neurohormones.[27,29,30] There is some overlap in transmitter molecules between vesicle types. Although sometime contested as an artifact of the fixation process, the evidence is now strong that there are two compartments in LDCVs, the halo with an electron-lucent region and the dense core with an electron-

dense under TEM due to the presence of a protein core.[31,32] The dense core is comprised of various soluble materials, such as a group of acidic proteins known as chromogranins and nucleotides. These proteins allow accumulation of relatively higher amount of neurotransmitters (0.7-0.9M) within the vesicles.[33] Hence, chromogranins are believed to be associated with neurotransmitters allowing reduction of the osmolality inside the vesicles, which could otherwise cause severe osmotic pressure to the membrane.[34–37] In addition to soluble and freely moving transmitters, the halo is made up of additional vesicular components including adenosine triphosphate (ATP), Ca^{2+} ions, and H^{+}. These molecules play an important role in maintaining an intravesicular pH of ~5.5.[38–40] Catecholamines can also interact with ATP in the halo to form weak complexes which might enhance the stability of these vesicles.[41,42] The structural distinction between the dense core and the halo results in different neurotransmitter concentrations in each region.[43–45] A significant fraction of neurotransmitters is tightly bound with the chromogranins in the dense core, whereas a smaller fraction of neurotransmitters is less strongly bound in the halo. This leads to differentiation in release dynamics from these two compartments.[46] Figure 4 shows TEM images of SSVs and LDCVs, dense core and halo.

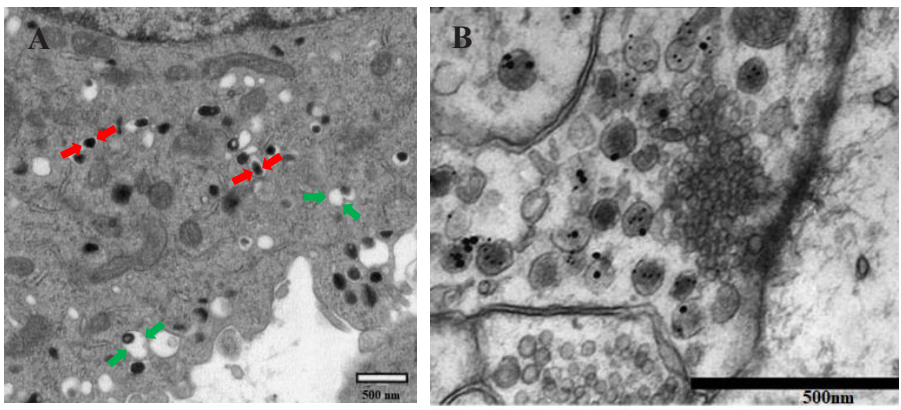

Figure 4. Representative TEM images from (A) single PC12 cells with the dense core (red arrows) and halo (green arrows), (B) a cluster of synaptic vesicles and dense cores from a simple axon terminal in rat amygdala. Reproduced with permission from Ref [36,47].

In the CNS, SSVs are highly abundant components compared to LDCVs, the latter accounting for only 1-2%.[48–50] However, a large number of LDCVs can be found in dopaminergic neurons, in specialized brain regions like the hypothalamus, and in the sympathetic neurons of the PNS.[50] Outside the central nervous system, LDCVs can also be found in neuromuscular junctions of for example the fruit fly *Drosophila* and mammalian neuroendocrine cells, such as chromaffin cells in the adrenal glands, and enteroendocrine cells from the crypt epithelium.[51–53] SSVs and LDCVs exhibit distinct properties, different speed and latencies, for release fusion upon Ca^{2+} stimulation. Nevertheless, regulation mechanisms of these two classes of vesicles share converse features which is mentioned in the following section.

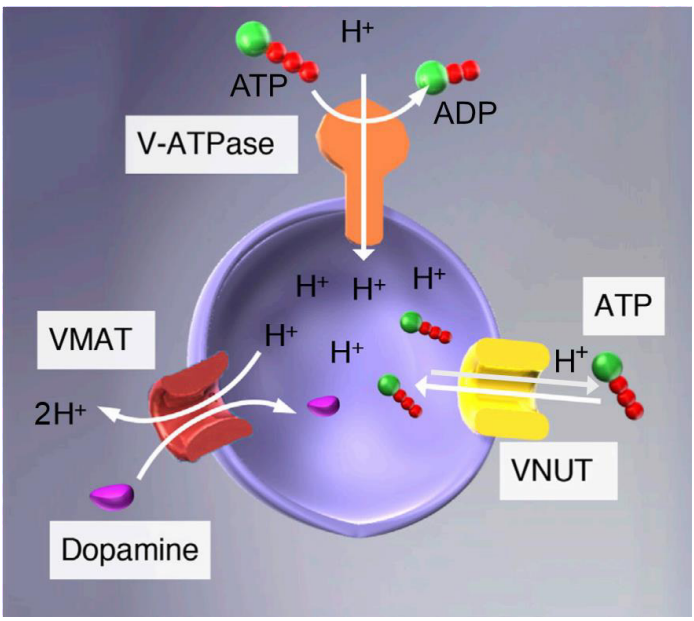

Figure 5. Schematic depiction of a secretory vesicle with three different transporter proteins, including V-ATPase, VMAT and VNUT. Reproduced with permission from Ref [54].

A large number of various membrane-bound proteins are found on a single vesicle. There are three main transporter proteins contributing to maintain the regular function of vesicles. These are the vesicular nucleotide transporter (VNUT), VMAT, and vacuolar-type H^+-ATPase

(V-ATPase) and their features are illustrated in Figure 5. The V-ATPases are ATP-dependent proton pumps localized at the plasma membrane in a variety of eukaryotes.[55,56] Many studies have suggested that V-ATPase plays a vital role in preserving an acidic intravesicular pH~5.5 and neutral cytoplasmic pH~7.4. The pH gradient across the vesicle membrane is achieved by utilizing the energy harvested from hydrolysis of ATP to adenosine diphosphate (ADP).[57] The acidification of the vesicle lumen is not only crucial to maintain the aggregated form and optimal binding capacity of chromogranins with pI range of 4.6 to 5, but also important for the active transport of neurotransmitters from the cytosol.[58] Additionally, the other two transporter proteins, VMAT and VNUT, utilize a pH gradient to transport ATPs and monoamine molecules such as dopamine, respectively, into the vesicular lumen. The function of VNUT is to import nucleotides into vesicles by pumping in one ATP molecule per proton pumped out.[59] VMAT can drive the uptake of one cytosolic monoamine in exchange for two lumenal protons expelled. There are two isoforms of VMAT, VMAT1 and VMAT2, which have been reported up to date. Despite belonging to the same family, the two transporters exhibit many different biological functions. Both transporters are responsible for the uptake of cytosolic monoamines such as dopamine, epinephrine, norepinephrine, and serotonin into vesicles, however, VMAT2 is capable of transporting histamine.[60,61] VMAT1 is present in LDCVs of neuroendocrine cells, including chromaffin and enterochromaffin cells while VMAT2 is prominently expressed in neurons of the sympathetic nervous system, and aminergic neurons in the enteric and the CNS. VMAT1 shows a slightly lower affinity, approximately three to fourfold, for most biogenic amines than VMAT2 does.[62] Moreover, VMAT2 has a higher turnover number than VMAT1. Hence, it is an important feature to explain the fast and slow secretion cycles of neurotransmitters in neurons and endocrine cells.[63]

2.2 The mechanisms regulating the exocytotic pathway and different modes of exocytosis

The release of vesicular content from secretory vesicles is called exocytosis. The process plays an essential role in maintaining proper organelle and cell functions, including brain activities and endocrine functions.[64] Illustration of vesicular trafficking mechanism involving exocytosis in the nerve terminal is shown in Figure 6.

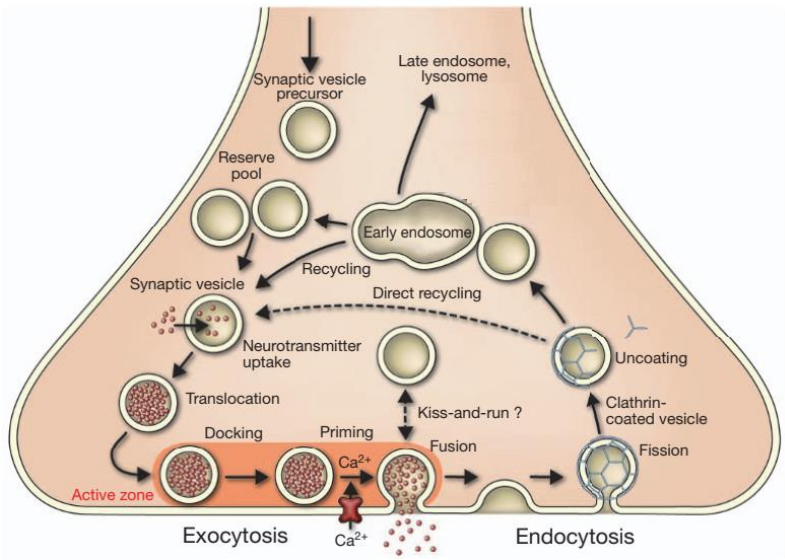

Figure 6. Schematic representation of the life cycle of a secretory vesicle in a synaptic terminal. Reproduced with permission from Ref [65].

Vesicles are generated from endoplasmic reticulum and trans-Golgi network of the Golgi apparatus. They cluster together in a reserve pool but only a small portion of these vesicles (~ 1-2%) are readily releasable.[66] Interactions between vesicles and components on the plasma membrane, or docking, is believed as the first step for exocytosis. Although many proteins have been identified to be involved in this step, the docking mechanism is not yet well established. The following step is called priming in which vesicles wait for a signal from rapid influx of Ca^{2+} ions to initiate the secretion. After exposure to the

trigger signal, a fusion pore is opened to allow neurotransmitters to diffuse into the extracellular environment. These three steps, docking, priming, and fusion, generally involve a variety of proteins to disrupt the intrinsic stability of the vesicle and the plasma membranes. The process is mainly driven by the soluble N-ethylmaleimide-sensitive-factor (NSF) attachment protein receptor (SNARE) proteins, including synpatobrevin, a vesicle bound (v-SNARE) membrane protein, and two plasma membrane proteins, syntaxin and SNAP25, or known as target SNAREs (t-SNAREs). Another protein from the Sec1/Munc-18 family, Munc-18, is capable of binding to syntaxin to induce the formation of the SNARE complex that pulls the vesicle and plasma membrane close to each other before fusion. Once vesicles are primed, synaptotagmin, a vesicle anchored protein, triggers the initiation of a fusion pore in response to the opening of Ca^{2+} channels on the plasma membrane.[67] In addition, synaptotagmin can also bind both SNAREs and phospholipids, which is suggested to accelerate the full SNARE zippering.[68] As the fusion pore opens, it allows neurotransmitters to escape and fuse into the synaptic cleft. After undergoing exocytosis, the reuptake or breakdown of chemical messengers is mediated by specific receptors and enzymes on the plasma membrane. Thereafter, vesicles are recycled via a process called endocytosis and the SNARE complex needs to be disassembled by ATPase NSF protein. Endocytosis can be classified into two different pathways, fast or slow mechanisms. Rapid rates of endocytosis have been indicated in the nerve terminal, which occur within several seconds, and kiss-and-run vesicle turnover has been suggested as a faster mechanism.[69,70] Clathrin-mediated endocytosis is thought to happen slowly with a time constant of seconds to minutes.[71]

Although the concept of the secretory vesicle recycling was introduced in the early 1970s, the destiny of these vesicles after fusion continues to be a matter for investigation and debate. It was postulated that vesicles could rapidly release all neurotransmitters and proteins and flatten the vesicle membrane into the plasma membrane. This full release mode was believed to be the only mechanism for exocytosis. However, transient capacitance of plasma membrane in mast cells was first observed from patch-clamp measurements. The detection of

flickering changes in the cell surface also suggested the formation of reversible fusion.[72] Additionally, the characteristic of the fusion pore has been validated by amperometry, which quantified low levels of the transmitter release during capacitance flickering. Altogether, these observations led to the indication of a second mode of secretion known as kiss-and-run.[73,74] In this mode, an initial fusion pore is relatively small and stays open for a very short time, the vesicle releases a small amount of its content and closes. A variation of release models between the full release and kiss-and-run mode have been reported.[43,75,76] Consequently, a third mode of exocytosis called partial release, an open and closed mechanism, has been proposed and suggested to be the most dominant mode during general exocytotic processes.[77] A small fusion pore expands and then remains open for a longer time, but not completely, in the partial release mode compared to kiss-and-run. This results in a larger number of transmitter molecules being secreted from the vesicles. Illustrations of these three release modes are depicted in Figure 7. Another variation of exocytotic release, known as the flickering mode, has been found in neurons where the release of chemical messengers is regulated via rapid flickering of the fusion pore.[78,79]

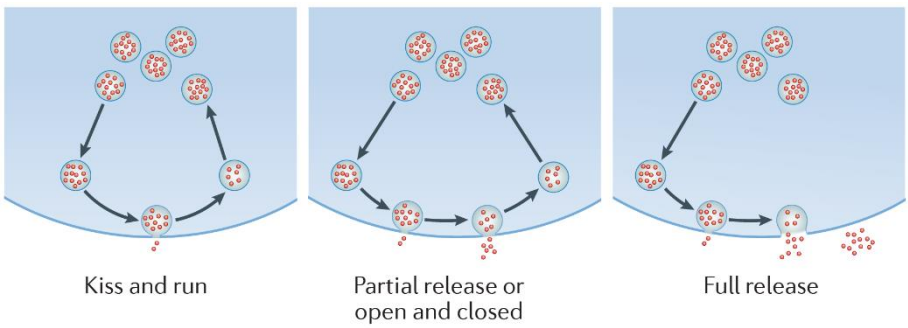

Figure 7. Three different modes of exocytosis: full release, partial release, and kiss-and-run. Reproduced with permission from Ref [80].

The plasticity of neuronal communication, involved in the formation of learning and memory, can be sorted into pre- and postsynaptic mechanisms.[81,82] One of the most important pathways of presynaptic plasticity is related to the alteration of amount of transmitter released

per vesicle.[61,64] The discovery of partial release as an exocytotic model provides more possibilities to study the regulation in the fusion process of exocytosis. Due to the high order complexity of vesicular secretion, many studies have been carried out to characterize vesicular structures under effects of pharmaceutical treatment or cellular activity.

2.3 Extracellular vesicles and their biogenesis

In addition to conventional synaptic neurotransmission, neural cells can communicate via the release of extracellular vesicles (EVs), which participate in manipulation of neurobiological functions including synaptic plasticity.[83] EVs are a cluster of heterogenous nanometer sized organelles secreted constitutively into the extracellular environment by a variety of organisms and almost in all cell types.[84–86] They are conventionally classified into two main groups: exosomes and ectosomes, depending on their biogenesis. The term exosome was adopted to describe the formation of vesicles generated from intracellular endosomes and released by reticulocytes in 1987.[87] Exosomes are originally produced from multivesicular bodies with an average diameter of 30-150 nm.[88–90] Ectosomes, or microvesicles, are generally referred to as 100-1000 nm vesicles derived by direct budding from the plasma membrane.[91] Small EVs with an approximate diameter of 50-100 nm are abundantly found in biological fluids. Medium sized EVs, 200-800 nm in diameter, are also present in smaller amounts. The least abundant population of EVs are large EVs, more than 1μm in size, including apoptotic bodies, large oncosomes,[92] secretory autophagosomes,[93] cytoplast,[94] migrasomes,[95] and exophers.[96]

Exosomes and ectosomes employ different mechanisms of biogenesis; however, both involve membrane trafficking processes. The formation of these EVs is illustrated in Figure 8. Exosomal vesicles are generated via the endosomal pathway. Early endosomes usually take place outside the cytoplasm whereas late endosomes occur inside the cytoplasm, near the nucleus. Intraluminal vesicles are accumulated in the lumen of endosomes during their maturation. The intraluminal vesicles are generated by the inner membrane of endosomes and stored in multivesicular bodies. Many multivesicular bodies play a primary role

as an intermediate to ensure the general degradation in the lysosomal pathway. In most cells, they can undergo fusion with lysosomes, which contain lysosomal hydrolase, resulting in the discharge and digestion of their intraluminal vesicles in the lumen of lysosomes. By contrast, organelles with hallmarks of multivesicular bodies, such as tetraspanins, lysosomal-associated membrane proteins, and other cytosolic proteins, can liberate their content into the extracellular milieu and fuse with the plasma membrane.[97] Exosomes have been found to facilitate myelin formation, neurite growth and neuronal survival in the CNS.[98–100] Additionally, pathogenic proteins including beta amyloid peptide and alpha synuclein have been identified in exosomes secreted from the CNS.[101,102]

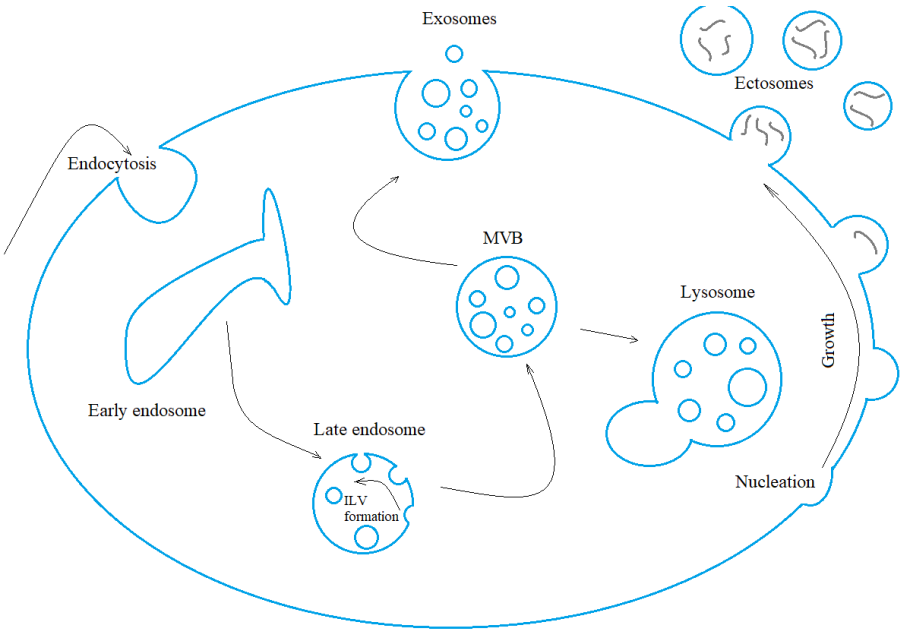

Figure 8. Schematic representation of biogenesis of two different types of EVs.

In contrast to exosomes, ectosomes are derived directly by an outward budding at the plasma membrane. Therefore, they also contain similar proteins as the cytosolic and plasma membrane, including tetraspanins which are known to cluster on surface of the plasma membrane. The amount of tetraspanins has been determined to be 100-fold higher in the

vesicles compared to the cell lysate.[103,104] Other common proteins found in these organelles are cytoskeletal proteins, heat shock proteins, integrins, and proteins containing post translational modifications, such as glycosylation and phosphorylation.[105–107] The lifespan of ectosomes is much shorter than exosomes and intracellular ectosomes can survive only during their generation.

Secretion of EVs was initially regarded as membrane debris to eliminate obsolete compounds from cells with no real biological significance.[108,109] However, both types of EVs play pivotal roles in cellular physiology and pathology as they have the ability to package active cargo containing proteins, lipids, and nucleic acids, and transport it to another cell that leads to the alteration of the recipient cell's function.[110] Although exosomes and ectosomes possess different mechanisms of biogenesis and secretion, both involve membrane trafficking processes. The two classes of EVs have similar intracellular mechanisms in which their components are sorted in their membrane of origin into small cargos. However, the formation of exosomes depends on the fusion of multivesicular bodies with the plasma membrane. The accumulation of biological materials is modulated by the cytosolic endosomal sorting complex required for transport (ESCRT) machinery from the plasma membrane and endosomes to the budding intraluminal vesicles.[111–113] There are four ESCRT complexes, each containing several subunits, and they play distinct roles not only in the biogenesis of intraluminal vesicles but also in protein sorting, particularly for ubiquitinated cargos.[114] ESCRT-0, can sequester and interact with the ubiquitinated proteins, while ESCRT-I, II initiates local budding from the endosomal membrane. These complexes are vital for cargo sorting. ESCRT-III takes part in protein deubiquitination and the formation of intraluminal vesicles to become exosomes. Intraluminal vesicles remain within multivesicular bodies for a considerable time, which leads to the delayed release of exosomes. The exocytotic fusion of exosomes, like that in conventional exocytosis of secretory vesicles but without any stimulation, is driven by a SNARE complex that includes vesicle associated membrane protein 7 (VAMP7) and the v-SNARE associated with most endosomes.[115,116] After fusion, the limiting multivesicular body membrane merges with the endosomal recycling system whereas

the intraluminal vesicles escape from multivesicular bodies as exosomes to the extracellular environment.[117,118] On the other hand, the shedding of ectosomes causes the removal of small portions of the plasma membrane. Ectosomes can accumulate larger numbers of cargo proteins in their lumen compared to intraluminal vesicles. The ESCRT machinery is also involved in the assembly and budding of ectosomes, and ESCRT-III is crucial for the pinching off and secretion processes.[119] Besides ESCRT, binding between cytoplasmic proteins and the plasma membrane is required for the assembly of ectosomes. Interestingly, these proteins are specific for the interaction of ectosomes with the plasma membrane and are not present in intraluminal vesicles assembly at multivesicular bodies.[120,121] Due to direct budding from the plasma membrane, the release of ectosomes does not require exocytosis. On release, some EVs do not remain intact and break down and release their contents into the extracellular space. In many cases, EVs discharge their vesicular components to the recipient cell cytoplasm by direct fusion with the plasma membrane.

In recent years, various studies have been carried out to investigate the extraordinary therapeutic potential of EVs to understand the underlying molecular mechanisms associated with EVs biogenesis, uptake, and biochemical properties in intercellular communication. EVs are considered to be promising targets in the diagnosis and therapy of diseases, particularly cancer. Moreover, the greatest interest of EVs is their role in advanced medicine, whereby they can be employed to identify biomarkers, improve diagnosis, and deliver bioactive molecules of drugs and human gene therapies.[122–125]

Due to the high complexity of conventional and unconventional secretion, it is necessary to develop simple biological models to address different aspects related to cellular exocytosis. In the following section, cell models and other common biological platforms are mentioned in detail.

CHAPTER 3. Biological models

The CNS is considered to be one of the most complicated and captivating organs in mammalians. For centuries, neuronal cells, the primary functional units of the CNS, have attracted the attention of many scientists because many of the neurodegenerative diseases and disorders are associated with their functional deficits.[126] However, the human brain and living neurons are not easily approachable either *in vitro* or *in vivo*. Thus, simplified biological systems such as animals or different cell types are employed to overcome this pitfall. This chapter includes detailed information about the two endocrine cell models, pheochromocytoma (PC12) cells and chromaffin cells used in this thesis, and a brief introduction of other prevalent biological models.

3.1 Chromaffin cells

Chromaffin cells are found in the adrenal glands, endocrine organs located in the upper region of the abdomen the above the kidneys. The adrenal medulla is commonly originated from rats, mice, and cows but sometimes it can be obtained from other sources such as guineapig, cat and even human.[127–129] Chromaffin cells are typically cultured as primary cells and they can express a variety of receptors and ion channels depending on the origin of animals.[129,130] There are two major classes, either epinephrine or norepinephrine cells based on the secretion of neurotransmitter type.[131] It is because of the presence of an additional enzyme PNMT that helps to induce the conversion of norepinephrine to epinephrine. A mixed population of epinephrine and norepinephrine cells is present in cultured chromaffin cells.

High extracellular K^+ concentrations can directly cause the depolarization of the chromaffin cell membrane and open the Ca^{2+} voltage channels which evoke catecholamine secretion. In chromaffin cells, catecholamines (mainly contain norepinephrine, epinephrine) are stored in LDCVs, also called as chromaffin granules. These granules contain a variety of other substances, such as peptides, including encephalin and neuropeptide Y, and proteins, chromogranins and secretogranins.[131] Chromogranins, or granins, are the main protein

making up the intravesicular matrix, accounting for more than 80% of the soluble protein in the intracellular environment, and it is capable of binding catecholamines to concentrate large amount of amines without changing the vesicle size.[132] There are three classical types of granins, consisting of chromogranin A, chromogranin B or secretogranin I, and chromogranin C or secretogranin II. They can also act as sorting proteins to regulate the secretory pathway which contributes to the formation of vesicles. Another essential aspect of chromogranins is their ability to bind Ca^{2+} with low affinity but high capacity (32–93 mol of Ca^{2+}/mol).[133] They can form aggregates at low pH in the presence of Ca^{2+}. In the trans Golgi network area with mild pH and high amount of Ca^{2+}, chromogranins tend to link to each other or other granule constituents such as ATP, catecholamines, or other proteins. This is how the dense cores can be assembled and recognized as electron-dense content in electron micrographs. Taken together, the chromogranin family is considered to be a pivotal modulator for the regulated secretory pathway and DCVs biogenesis in neuroendocrine cells.

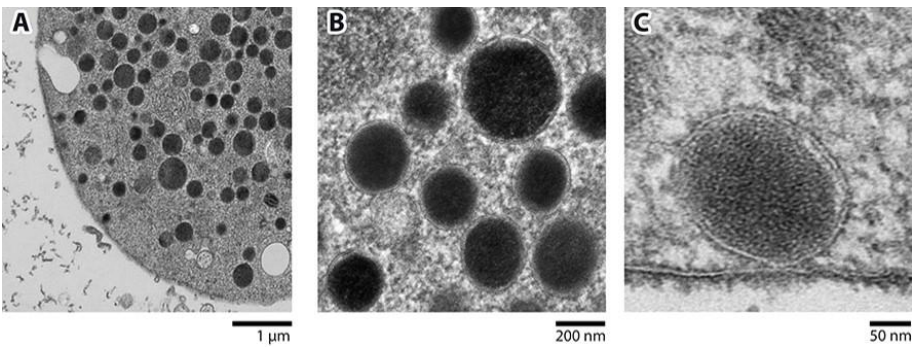

Figure 9. TEM images from a primary bovine chromaffin cell (A), preserved large dense core vesicles (B), and a large dense core vesicle at the docking stage to prepare for exocytosis (C). Reproduced with permission from Ref [134].

LDCVs are specialized secretory vesicles and have been exploited as a model for in-depth study of dense core biogenesis as about 13.5% of the cytoplasmic volume of the chromaffin cell is constituted by these vesicles.[135,136] Each chromaffin cell isolated from the adrenal medulla has about 10 000 vesicles. Figure 9 illustrated a bovine chromaffin cell

with preserved LDCVs and a LDCV docked at the plasma membrane potentially about to release.

As a primary cell line, chromaffin cells more closely resemble healthy cells *in vivo*. LDCVs in chromaffin cells are much larger than those in PC12 cells and contain more catecholamine neurotransmitters, so this makes it simpler to quantify vesicular content and chemical messenger release by electrochemical techniques. However, since animal tissues are comprised of several different cell types, the preparation and culture of this cell line is much more challenging compared to the use of immortal cell lines. Primary cultures have a short lifespan, several days, and the number of cells available is also limited and reduced over time. Moreover, another important consideration is related to ethical approvals when performing experiments with animals. Finally, heterogeneity, batch to batch differences between adrenal glands, is also an important factor that needs to be considered for data analysis and interpretation.

3.2 PC12 cells

Together with chromaffin cells, PC12 cells, an immortalized neurosecretory cell line, are one of the most commonly used cell models for the study of neuronal transmission. PC12 cells are an established cell line derived from a pheochromocytoma cyst of the rat adrenal medulla. This cell type was first isolated in the Lloyd Greene lab and published in 1976.[137] These cells can synthesize, store, and secrete appropriate quantities of catecholamines.[138] Unlike many other chromaffin cells, epinephrine is undetectable in PC12 cells due to the lack of PNMT, the main enzyme responsible for the conversion of norepinephrine to epinephrine. However, an abundance of dopamine has been determined in many sublines of PC12 cells by using high pressure liquid chromatography and capillary electrophoresis.[139–141] A small amount of norepinephrine can also be found in some cells. These two neurotransmitters are stored in LDCVs, which are generally smaller and contain lower concentration of catecholamines than those in chromaffin cells.[137] Moreover, some studies have reported the probabilities of two distinct classes of dense core vesicles, which are

different in size and vesicular content.[142,143] Alongside catecholamines, acetylcholine is also present within the cellular environment inside small clear vesicles in PC12 cells.[144] Figure 10 shows some TEM images of PC12 cells with electron dense LDCVs.

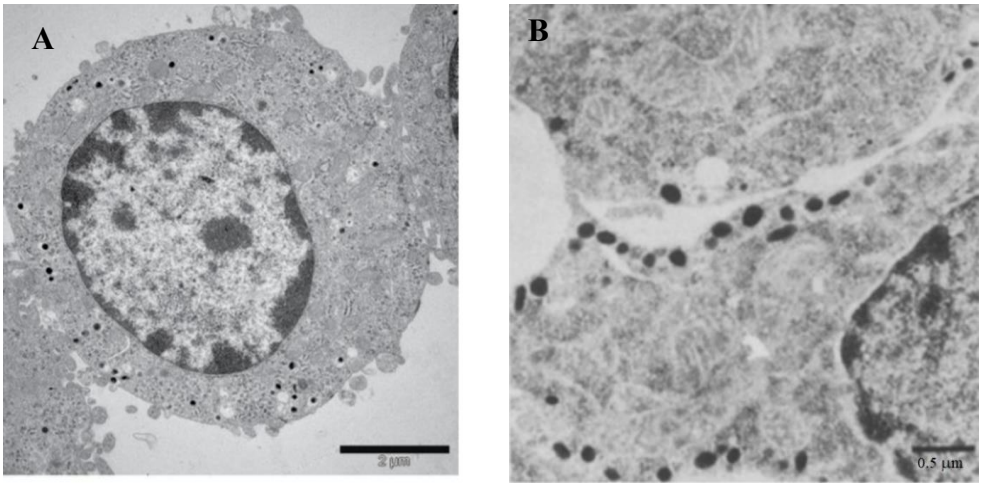

Figure 10. TEM image of a PC12 cell and visible LDCVs (A) and docked LDCVs can be observed underneath the plasma membrane waiting for the signals to start fusion (B). Reproduced with permission from Ref [145].

There are two commercial variants of PC12 cells, well-attached adherent, and suspension cells. The PC12 cells in suspension grow as a cluster of floating cells with small and irregular shapes. They can develop neurite outgrowth resembling sympathetic ganglion neurons in the presence of nerve growth factor (NGF) and this process also requires the cells to be adherent. In contrast, adherent cells can be grown on plastic surfaces like culture flasks/dishes or an appropriate coating to form monolayers. Some widely used reagents for coating are poly-L-lysine (PLL), poly-D-lysine (PDL), fibronectin, laminin, and collagen (type I and IV).[146,147] Despite taking more time and higher cost, adherent PC12 cells are more functional for certain types of experiments, which demand the cells to be immobilized, *e.g.,* electrodes need to approach single cells on their surfaces in some electrochemical

methods. Thus, the selection of proper PC12 cell types is dependent on experimental conditions and hypotheses.

One of the most conspicuous features of PC12 cells is the capability to differentiate into sympathetic nerve-like cells under the influences of NGF. Additionally, they can synthesize acetylcholine and can make cholinergic synaptic contacts like the primary cultures.[148] Acetylcholine is not only a neurotransmitter involved in cognitive functions but also takes part in learning and plasticity. There are two types of acetylcholine receptors: cholinergic M, or muscarinic, receptors and cholinergic N, or nicotinic, receptors. Both families of receptors contribute to regulate the cognitive functions, particularly nicotine receptors have great impact in Alzheimer's disease by interactions between their subtypes and amyloid beta.[149,150] N-methyl-D-aspartate receptors, glutamate-gated cation channels with high calcium permeability, are present on the PC12 cell membrane. These receptors can interact with neuronal proteins such as amyloid β-peptide/amyloid precursor, α-synuclein protein, and tau protein.[151,152] Consequently, the neurosecretion process can be manipulated via interfering catecholamine synthesis, vesicular and plasma membrane uptake.[153,154] Altogether, the PC12 cell line possesses many especially important and striking features to study pathological molecular mechanisms of dementia such as Alzheimer and Parkinson's disease.

For a half century, PC12 cells have been exploited as useful model systems in neurobiological research, including neurotoxicity, neuroinflammation, neurotransmitter secretion, and differentiation methods. These cells allow easy manipulation and are capable of continuous replication as they are tumour derived or known as immortal cells. But the major disadvantage of the cell line is that they can proliferate indefinitely and sometimes generate unusual gene patterns, which cannot be found in any cell types. Furthermore, cell characteristics can also be changed after multiple passages and become more different from original ones. This leads to deviations and difficulties in comparing between different data sets. Therefore, it is crucial to regularly evaluate the properties of cultured cells and not use cells that have been propagated too many times.

3.3 Other models

In addition to the two major cell types described above that have been employed for experimental study in this thesis, there are several biological models available for neuroscience research. In vitro models have been established to provide a simplified system to understand neuronal cell development, biological function, and interaction with other cells. Immortal cell lines are widely used in laboratories across the world because they are relatively easy to maintain, commercially available and can replicate indefinitely. Some examples of these common cells are Chinese hamster ovary (CHO) cells, human embryonic kidney 293 (HEK-293) cells. Another popular cell model is primary cells, those that are isolated directly from living tissues or organs. They preserve many similar characteristics and functions as the tissue of origin and therefore closely represent the *in vivo* state and exhibit normal physiology. There are several cell types in research such as epithelial cells, endothelial cells, hematopoietic and mesenchymal stem cells. In addition, three dimensional neuronal organoids derived from stem cells are providing exciting new models for CNS studies *in vitro* as a more precise representation can be carried out in a highly specialized manner.

The use of model organisms or animals is more common in the behavioural sciences, providing insights into the structure and function of the nervous system. Mice and rats are the most widely used due to their genetic similarities to humans and a variety of genetic manipulations available. Fruit flies, *Drosophila melanogaster*, have been exploited as a model organism in the field of genetics and development biology as they contain relatively simple genome compared to mice and rats and short life cycle for reproduction, taking 10 to 14 days to produce a new generation. Furthermore, zebrafish, *Danio rerio*, and nematodes or round worms, *Caenorhabditis Elegans*, are also used for neuronal development and social behaviours.

Generally, the above mentioned biological models offer different levels of complexity, allowing one to address various aspects of neuroscience research. Depending on the specific research questions and goals of

study, the choice of appropriate model is determined, based on advantages and disadvantages of each modelling system.

CHAPTER 4. Analytical methods to characterize vesicular structures

Vesicles are one of the main organelles responsible for cell-to-cell communication due to their crucial roles in physiological and pathological processes. Over recent decades, understanding the relationship between vesicle structure and function has attracted widespread interest. Therefore, various technologies and methods have been employed to determine different characteristics of vesicles. This chapter aims to introduce in detail different electrochemical approaches that have been developed to investigate physiochemical properties of vesicles such as size distribution, vesicular content, neurotransmitter release and concentration of metabolites or transmitters in vesicles.

4.1 A brief introduction of electrochemical analysis to investigate vesicular properties

In the 1950s, measurement of the spontaneous synaptic potentials on the transmission at the frog neuromuscular junction led to the establishment of the quantal release of neurotransmitter.[155,156] Subsequently, these experimental observations led to formulation of the quantum hypothesis, in which transmitter release from vesicles was quantized, by Martin in 1966.[157] Improvement of patch clamp techniques was successfully implemented to record a tiny area of the plasma membrane, with capacitance changes caused by the exocytotic and endocytotic processes.[158]

In the 1990s, amperometry with carbon fiber microelectrodes was first introduced to monitor chemical release from bovine adrenal chromaffin cells *in vitro* by the Wightman lab.[159] Together with cyclic voltammetry, the temporally resolved current spikes were revealed due to exocytosis of individual vesicles.[160] Both electrochemical techniques have been employed to detect vesicular events and characterize exocytosis.[161,162] In 2015, the two methods named vesicle impact electrochemical cytometry (VIEC) and intracellular vesicle impact

electrochemical cytometry (IVIEC) were reported by the Ewing group.[163,164] These can be used to quantify vesicular content both outside and inside cells, respectively.[9,10] Development of VIEC with carbon nanopipettes (CNPs) has allowed simultaneous determination of vesicular size and their content with these confined electrodes (**paper I**). Subsequently, using a similar method, IVIEC with CNPs was utilized to quantify the catecholamine content and release kinetics of vesicles in bovine chromaffin cells after exposure to L-DOPA (**paper II**).

4.2 Single cell amperometry

4.2.1 Principles of amperometry

Amperometry was initially employed to measure secretion in whole tissue samples, *e.g.*, exploring neurotransmitter release in the brain, in the biological science.[165,166] This method has been used to study the release of neurotransmitters and hormones in brain tissue and cerebral spinal fluid since the early 1970s.[166] Single cell amperometry (SCA) was first described in 1990 and soon became a powerful tool to study the mechanisms underlying vesicle secretion from cells.[167] The potential is in this case kept constant while the flow of current is measured. Any changes in current results from the oxidation of electroactive molecules present inside vesicles, which can provide valuable information regarding the mechanisms regulating this coordinated cell secretion.

In the modern version of the SCA technique, a microelectrode with a diameter of 5 – 10 μm, beveled at a 45° angle, is positioned in close proximity to the plasma membrane as shown in Figure 11A. The arrangement is regarded as a simplified illustration of a synapse since the electrode can be considered as a receiving cell. The electrode is held at a constant potential (~700 mV for catecholamine oxidation), the release of oxidable substances such as dopamine, epinephrine, and norepinephrine can cause a concomitant change in the current as a function of time. The secretion of these chemical messengers can be triggered with or without stimulation to measure release from secretory

vesicles or release of EVs, respectively. To measure release from secretory vesicles, an electrical or chemical stimulus needs to be applied in order to increase the intracellular Ca^{2+} concentration. This triggers the vesicle fusion initiating exocytosis. A glass pipette filled with stimulation solution can be set close to the cell surface for chemical stimulation (Figure 11A). Stimulation solution with a high concentration of K^+ is commonly used. Under resting conditions, cells are incubated in isotonic solution, which is similar to the extracellular environment. Hence, there is no current flow as no oxidable molecules are present at the electrode surface. The isotonic solution contains a high concentration of Na^+ ions and a low concentration of K^+ ions, whereas the stimulation solution is comprised of a high concentration of K^+ and a low concentration of Na^+. When the stimulation solution is applied to the cell surface, the cell membrane is depolarized due to the disruption of the ionic gradients across the membrane, and this initiates vesicle fusion. The released transmitters can diffuse close to the electrode surface, and these molecules are oxidized by transferring electrons to the electrode. This process results in a flow of current which can be measured and recorded as current transients for each exocytotic event.[159]

The charge for each event can be quantified to determine the number of moles *N* released. The charge Q, obtained from the time integral of the spike, is directly proportional to the number of released molecules during the event, as described in Faraday's law. In the equation, n is the number of electrons transferred during the reaction (n=2 for the oxidation of catecholamines) and F is the Faraday's constant (96 485 $Cmol^{-1}$).

$$N = \frac{Q}{nF}$$

In addition to the quantitative information, the amperometric spike also provides dynamic information about the release event. Different parameters of a single spike are indicated in Figure 11B. The half width ($t_{1/2}$) is the width of the peak at its half maximum amplitude and indicates the duration or the stability of the fusion pore. The rise time (t_{rise}) is the time measured for the spike to rise from 25% to 75% of the

height and represents opening duration of the fusion pore or the rate of pore expansion. The fall time (t_{fall}) is the time required for the spike to fall from 75% to 25% of the height and specifies the rate of diffusion intermixed with a possible closing of the fusion pore. The maximum current (I_{max}) is the maximum amplitude measured from the baseline to the highest point of the spike. I_{max} is not well understood yet in exocytosis, but probably indicates the fusion pore size during the release event.

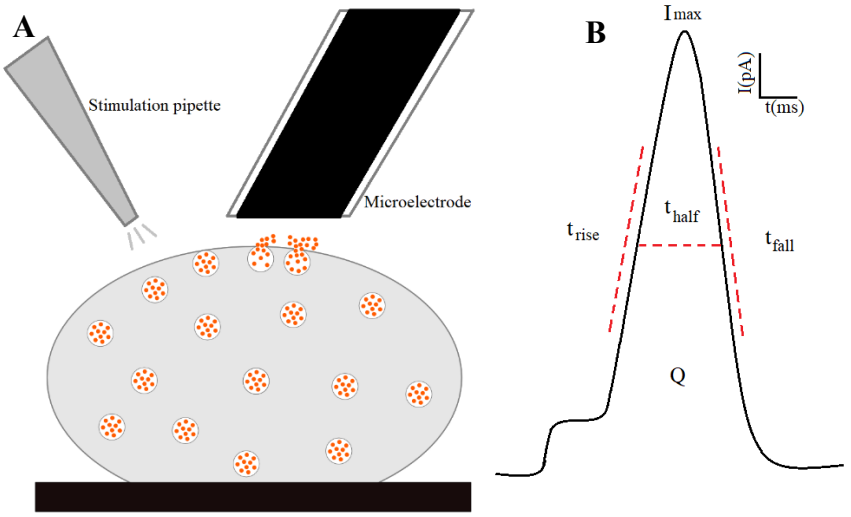

Figure 11. A schematic presentation of SCA measurement for measuring transmitter release from a single cell (A) and illustration of an amperometry peak with different kinetic parameters (B).

Amperometry is a non-invasive method with high sensitivity and high temporal resolution (sub millisecond). It is assumed that this technique does not affect normal function of the cell or the secretory responses. Another major advantage of amperometry over other techniques is the ability to provide direct quantification of secreted chemical molecules. However, amperometry is only applicable for oxidable molecules and unable to discriminate between different substances, which can be oxidized at the same potential. Another drawback comes from the limitation of the electrode size that is typically used, which causes the lack of spatial resolution. These obstacles can be solved by using

enzymatic biosensors for non-electroactive neurotransmitters[168] or arrays of smaller electrodes to enhance spatial resolution.[169–171]

4.2.2 Applications of SCA

Taking advantage of great temporal resolution and high sensitivity, as well as quantitative capability, amperometry has been shown to be a powerful technique in the study of vesicular secretion. It has been used to investigate exocytosis process in many cell types, including primary cultures, nerve cells, synapses, and other secretory cell models such as chromaffin cells, PC12 cells, mast cells, and pancreatic β cells.[172] Moreover, *Drosophila* is also considered as a typical model to examine the exocytotic release *in vivo* and octopamine release from the neuromuscular junction has been described in a live, dissected larval *Drosophila*.[173] Quantal release of catecholamines was observed from axonal varicosities of midbrain dopamine neurons containing SSVs[174] and on the surface of somas of cultured neurons.[175] The secretion of norepinephrine from single synapses has been successfully measured and reported.[176] In addition to exocytosis of secretory vesicles, amperometric detection has been developed for determination of exosomes and dopamine-containing exosomes are successfully detected from Hela and PC12 cells.[177] In a new twist of this area, neurotransmitters from single exosomes of chromaffin cells have been captured and quantified by amperometry as discussed in **paper III**. These studies also include discrimination between exosomes and other EVs showing the release of transmitter molecules via exosomes.

Furthermore, amperometry has been used to study the effects of various drugs on exocytotic release. The glycocalyx was reported as an important factor for controlling and hindering neuronal communication.[178] The effect of latrunculin A, an inhibitor of actin cross-linking, was investigated on exocytosis in PC12 cells.[179] Exocytosis is inhibited at a high concentration of tamoxifen while low levels of tamoxifen can stimulate the exocytotic process.[180] Zinc, present in neurotransmitter-containing vesicles with a high concentration and plays a key role in the CNS, changes vesicular structure, fusion pore, and the rate of neurotransmitter release.[181] These

papers mentioned above are only a few representative examples among tremendous applications utilizing amperometry as one of the outstanding techniques to measure the impact of exocytosis.

4.3 Vesicle impact electrochemical cytometry and intracellular vesicle impact electrochemical cytometry

4.3.1 Methodologies of VIEC and IVIEC

Information about content of vesicles must be retrieved to fully understand the mechanism of the vesicular release process. Flow vesicle electrochemical cytometry was the first method developed to quantify individual vesicular neurotransmitter content. The combination of capillary electrophoresis, microfluidics, and electrochemical detection allowed detection of single dopamine-containing liposomes.[182] In the hybrid device, isolated vesicles were separated according to their size and charge by capillary electrophoresis. Vesicular contents were released by the application of a sheath flow of lysis buffer and were measured by using end-column detection with a carbon fiber amperometry electrode. Furthermore, direct comparison of the total catecholamine content in vesicles versus the conventional amperometric release at single PC12 cells revealed that vesicles release only 40% of their entire neurotransmitter content during exocytosis.[183] Dopamine molecules were then counted at an average of 33000 per vesicle from mouse brain tissue, which is higher than the amount of transmitter content at cultured neurons during quantal release.[184] These findings indicated an interesting possibility that exocytotic release proceeds in an incomplete manner and strengthen the hypothesis of a partial vesicular release mode with only a fraction of quantal content expelled.

VIEC was then developed to eliminate the separation step and simplify quantification of vesicular content without addition of lysis buffer by the Ewing group in 2015.[185] In this method, vesicles were isolated from cells or tissues, but instead of separating vesicles before measurement, detection was obtained by placing a 33-μm disk-shaped carbon fiber electrode in a suspension of isolated vesicles. Vesicles adsorb onto the

electrode surface and consequently release their contents against the polarized electrode with the membrane opening via electroporation. The chemical cargo from a vesicle is oxidized and can be recorded as an amperometric spike due to the applied potential on the electrode. Rupture of each vesicle is represented by one spike in an amperometric trace. In the initial experiments, the most abundant distributions of current transients come from the rupture of single vesicles accounting for 86% of all amperometric spikes, leading to the ability to quantify single vesicle events.[185] The capability to study the effects of various drugs on vesicular structures without any interferences from other cellular organelles is considered to be an important advantage of this technique. However, interactions between vesicles and other intracellular compartments are also crucial to fully understand the behaviours of vesicles within their natural environment. Furthermore, vesicles with smaller size can be depleted and vesicular content might leak during the vesicle isolation process. Due to these limitations, it was important to establish an *in situ* method to evaluate the cellular response of vesicles without changing any environmental surroundings, in which the cell is treated as an intact system.

The intracellular version of VIEC, called IVIEC, was developed to directly measure neurotransmitter content in individual vesicles in the cytoplasm of live cells.[186] A nanotip conical carbon fiber electrode with 50-100 nm diameter obtained by flame-etching was employed to penetrate the cell membrane with minimal damage and expose the electrode surface to intracellular vesicles. The IVIEC technique is similar to the VIEC method as they are both based on the same principles. In the case of IVIEC, vesicles inside the cytoplasm are adsorbed on the electrode surface and subsequently rupture by electroporation to discharge their contents. This allows transmitter oxidation for quantification. Quantitative modelling of collection efficiencies from nanotips and disk-shaped electrodes demonstrates that the amount quantified by IVIEC is the same as that in VIEC.[187] Therefore, IVIEC can capture 100% of the vesicular content which provides a reliable method for measuring transmitter storage in single vesicles. The distribution of catecholamine content can be compared with the molecular distribution obtained from the exocytotic release

using SCA. It has been shown that about 64% of chemical messenger molecules was released in a vesicle during regulated exocytosis from PC12 cells.[186] Once again, the prevalence of the partial release mode has been shown, in which vesicles are not fully open to prevent discharge of their entire vesicular storage.

4.3.2 VIEC and IVIEC with open carbon nanopipettes

CNPs have been successfully developed for performing real time quantitative measurement within a single cell. Their diameters can vary from ten to a few hundred nanometers.[188–191] Fabrication of these nanopipettes utilizes quartz or borosilicate glass capillaries as the precursor. The selection of using quartz versus borosilicate is mainly defined by the pore size and properties of the inner surface for specified experiments. Although borosilicate is easy to manipulate, the soft nature of the glass makes it challenging to construct pipettes with diameters smaller than 80 nm. On the other hand, quartz is more durable and can be used to produce pipettes with various diameters including ultrasmall pipettes with orifice sizes down to 10 nm.[192] Additionally, a quartz pipette possesses significantly less noise than that made from any other glasses due to its low dissipation factor of ~10^{-4} and dielectric constant of 3.8.[193] Therefore, quartz is suitable for both intracellular single cell and electrochemical applications because of the low electrical noise and high mechanical strength.[194] In this thesis, quartz pipettes have been utilized to simultaneously investigate vesicle sizes and vesicular content at a single cell level.

The first development of carbon-based pipettes was reported by Kim and coworkers, who were able to accumulate multiple layers of carbon on the inside and outside of aluminosilicate pipettes by using catalytic carbon vapor deposition.[189] Four years later, Singhal *et al.* successfully fabricated CNPs with noncatalytic chemical vapor deposition with pore sizes ranging from 10 nm to 200 nm.[188] Capillaries were heated and pulled into two near-identical conical pipettes by using a laser pipette puller. A gas mixture containing a carbon source (methane) and a carrier gas (argon) was injected into the pipettes while placed in a furnace at 880-950ºC. Methane was decomposed into carbon at this high temperature and deposited as a several nanometer thick layer inside

these quartz pipettes with an open path in the middle, giving the name open CNPs. The deposition process can take place continuously over times of 30 minutes, allowing manipulation of fluid flow and ionic current sensing. CNPs can be used to simultaneously record the ion current through the pipette and the current derived from oxidation or reduction of chemical molecules on the carbon surface.

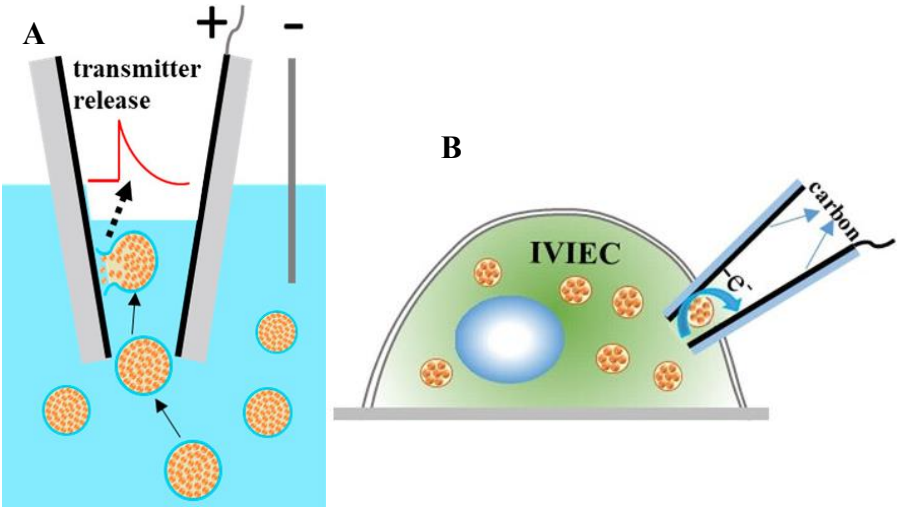

Figure 12. A schematic presentation of two different applications of CNPs to measure isolated vesicles by VIEC (A) and to directly quantify vesicular content in a single cell by IVIEC (B).

Open CNPs have been employed in the electrochemical determination of catecholamines.[188,195,196] A schematic of VIEC and IVIEC measurements with open CNPs is depicted in Figure 12. Isolated vesicles or intracellular vesicles are small enough to diffuse through the pipette orifice and release their content on the inner wall of the pipette. The CNP tip is dipped in vesicle suspension to carry out VIEC or inserted into the cytoplasm to carry out IVIEC. The thin layer of carbon makes it effective for vesicles to adsorb and rupture by electroporation. The oxidation of messenger molecules from individual vesicles are recorded as current transients and each spike represents the release of a single vesicle. The amount of catecholamine is integrated from the charge of the spike and based on the same principle as carbon fiber microelectrodes. The main advantage of carrying out measurements

with these two techniques by use of CNPs is the ability to simultaneously determine vesicular size and content. This is a significant advancement for mechanistic investigations of pharmaceuticals in exocytosis regulation at the single cell level.

4.3.3 Applications of VIEC and IVIEC

The ability to quantify the total neurotransmitter content in individual vesicles provides a powerful tool to understand fundamentals of cellular communication. Together with SCA, these two complementary methods, VIEC and IVIEC, have been employed to obtain a broader perspective of cell secretion. The release fraction from regulated exocytosis can be calculated by the amount of expelled over stored transmitter molecules. The fraction of release can be manipulated by a variety of pharmacological treatments as well as cellular activity. It is also valuable for quantification of partial release as a mode of exocytosis.

The first application of combined IVIEC and SCA was utilized to understand the partial nature of exocytosis.[186] Only 70% of vesicular content was released by comparing the number of catecholamine molecules in PC12 cells obtained from carbon fiber electrodes. This indicates that partial or "sub-quantal" release is the dominant mode of exocytosis. After L-DOPA exposure, transmitter storage and release were highly accumulated with almost equivalent levels, thus maintaining the same release percentage as the control. In a series of experiments, chromaffin cells were exploited to study the effects of L-DOPA on the vesicular structure by now using CNPs, and these results are included in **paper II**.

A variety of drugs have been used to study the regulation of partial exocytosis measuring vesicular chemical storage and chemical release. The DAT, a presynaptic membrane protein responsible for transportation of dopamine back into cells, can be inhibited by cocaine and methylphenidate.[197] Although these two compounds decreased both vesicular content and transmitter release during exocytosis, the fraction of neurotransmitter release from vesicles was manipulated differently. The fraction of dopamine release was reduced from 74% for the control

to 65% with cocaine treatment whereas the release fraction went up to 83% in the presence of methylphenidate. The importance of this result is that these drugs have opposite effects on cognition suggesting partial release might play a role in neuronal plasticity.[45]

4.4 Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

Mass spectrometry imaging (MSI) is a powerful tool which can visualize the spatial distribution of molecular species on a sample surface. A variety of analytes including proteins, peptides, metabolites, biomarkers, and lipids can be analyzed without the need for labeling. During MSI, a desorption or ionization probe, such as a focused ion beam or laser, is utilized to scan across the sample surface and acquires several spectra from different points to generate a chemical image. Molecules desorbed and ionized from each measurement position are subsequently separated and analyzed by a mass analyzer based on their mass-to-charge ratio. The intensity of each ion for each pixel point can be reconstructed to generate a chemical image. Therefore, the spatial distribution of specific ions can be visualized.

Several types of MSI techniques have been developed including secondary ion mass spectrometry (SIMS), matrix-assisted laser desorption ionization, and desorption electrospray ionization. Among these MSI approaches, SIMS is recognized as one of the most sensitive elemental analysis methods and can offer the highest spatial resolution ranging from micrometer to nanometer scale. The initial development of SIMS was proposed in 1960s by Raymond Castaing and George Slodzian.[198] In SIMS, the surface of a solid sample is bombarded and sputtered by using a primary ion beam. The mechanism of this process can be described as a collision cascade of the particles.[199] Since a primary ion with high energy strikes the sample surface, its energy is transferred to atoms of the surface and the surface molecules can gain sufficient energy to emit secondary ions. These secondary ions are collected and analyzed to obtain valuable information on elemental, molecular, and spatial distribution on the surface or within samples.

There are two operation modes of SIMS: static and dynamic. Static SIMS using a low intensity primary ion beam was developed by Alfred Benninghoven and coworkers, allowing the identification of both elemental and molecular information of surface species.[200] Conversely, dynamic SIMS utilizes a focused high energy primary ion beam to increase higher fragmentation efficiency and is usually preferred to determine the in-depth concentration of interested species.

ToF-SIMS is a standard technique for static SIMS in which secondary ions are separated according to their different velocities in the ToF mass analyzer. Most instruments employ a short pulsed primary ion beam to irradiate the sample surface and induce the formation of secondary ions. All these secondary ions are then accelerated by an electric field before entering the flight tube. The velocity of each ion is distinguished by its mass-to-charge ratio when traveling through the ToF mass analyzer. Heavier ions take longer time to reach the detector whereas lighter ions travel faster and arrive at the detector earlier. However, different initial kinetic energies from the ions with the same mass can cause a broadening of the ion packet, which leads to poor mass resolution due to the broad energy distribution of secondary ions.[201] One way to enhance the mass resolution is the insertion of a two-stage ion mirror system, also called a reflectron.[202] The secondary ions are reflected towards the detector by an electrostatic field from the ion mirror. Therefore, high kinetic energy ions have more velocity and penetrate deeper into the mirror compared to ions with lower energy. All secondary ions with the same mass but different initial kinetic energies can reach the detector at the same time, resulting in a better mass resolution.

The surface sensitivity of ToF-SIMS allows localization as well as identification of interested molecules on the cell membrane. Together with electrochemical techniques, ToF-SIMS imaging has been employed to investigate the alterations of cellular lipids that might be related to regulation of exocytosis in **paper IV**.

CHAPTER 5. Summary of papers

This thesis was mainly focused on vesicular content and release characteristics to enhance our understanding of the mechanisms of vesicle secretion. The applications of electrochemistry techniques were included to investigate chemical messengers in individual vesicles. The combination of open CNPs with these methods enables us to correlate vesicular size and the number of neurotransmitters from single vesicles in a living cell. These studies provide comprehensive insights of chemical communication at the single cell level and reveal complexities of vesicular exocytosis.

In **paper I**, open CNPs with different diameters between 50 and 600 nm were employed to perform VIEC measurements on isolated vesicles from adrenal chromaffin cells. This allowed me to simultaneously determine the number of transmitter molecules and vesicular sizes. The results indicated that the vesicular content increases with vesicle size. The dense core size and release dynamics have the same relation with the vesicle size, implying that the release speed of each event depends on the vesicular dense core size. In addition to VIEC, open CNPs also provide a promising IVIEC method to quantify the vesicular content for the in situ intracellular environment.

In **paper II**, the vesicular content and release kinetics of specific vesicle populations was quantified by the IVIEC technique with open CNPs in adrenal cells. The narrowed distribution of vesicle sizes and content was achieved through use of a 100-nm pipette orifice to obtain reliable information regarding catecholamine concentrations. The catecholamine concentrations in the intracellular environment were determined to be 0.23-1.1 M. L-DOPA, the biosynthetic precursor of catecholamines and a clinical drug for the treatment of PD, was utilized to explore the mechanistic investigations of L-DOPA in the regulation of exocytosis. The alterations of vesicular structure, molecular number and concentration after L-DOPA exposure were quantified simultaneously. Altogether, it was shown that IVIEC with open CNPs can be used for quantification of single vesicular catecholamine concentrations in living cells and opens a new avenue for the

mechanistic study of pharmaceuticals in the regulation of the exocytotic process at the single cell level and in small living organisms.

Apart from intracellular or secretory vesicles, the dynamic release of single exosomes, one of the main types of EVs, from a single living cell was unraveled in **paper III**. Amperometry with microelectrodes was introduced to detect electroactive catecholamines encapsulated inside exosomes. This method can also discriminate catecholamines inside exosomes and those released from intracellular LDCVs via exocytosis. Direct, real-time, and selective detection of single exosomes was obtained by controlling the cell-electrode distance as well as application of a stimulation to elicit release. As catecholamines are also neurotransmitters, the findings suggest a new mechanism of chemical communication at the fundamental level, changing the conventional view of exocytosis process.

Paper IV is outside the theme of the other papers as it presents the use of amperometry and mass spectrometry to examine the effects of ketamine on exocytosis and vesicular content. Here, subanaesthetic and anaesthetic doses of ketamine were used to investigate catecholamine storage and release in pheochromocytoma (PC12) cells. IVIEC and SCA were applied to show that the intravesicular contents and number of exocytotic events increased and that the fraction of release decreased after treatment by subanesthetic ketamine. Then ToF-SIMS was used to show that cellular lipids change following acute ketamine administration relative to control cells with an increased proportion of low-curvature lipids and a decreased proportion of high-curvature lipids. The conclusion was that ketamine modulates dopaminergic function with a dose-dependent pharmacologic action by interacting with different proteins and changing lipid composition. This work might be helpful in our understanding of the activity of the dopaminergic system in drug abuse. It could also be useful in understanding ketamine as an anesthetic or antidepressant molecule.

CHAPTER 6. Concluding remarks and future outlook

In multicellular organisms, cell to cell communication plays a vital role for survival and chemical signalling is the most abundant type of the communication. Cellular chemical communication can be sorted into two general forms: intercellular signalling between cells governed by EVs and intercellular signalling regulated by secretory by intracellular vesicles fusing with the cell membrane in the process of exocytosis. This process involves secretion of chemical signalling molecules from vesicles into the extracellular space. Conventional exocytosis is tightly mediated by both types of secretory vesicles, LDCVs and SSVs, and regulatory mechanisms are generally studied with model cells containing LDCVs. Aggregated chromogranins are the main components of the dense core and they can bind with neurotransmitters, whereas the vesicular halo contains freely moving transmitters. The pathologies of many neurodegenerative diseases and memory disorders are related to regulation of exocytosis, including vesicular structures and release dynamics of the dense core matrix. On the other hand, EVs, particularly exosomes which originate from the exocytosis of multivesicular bodies, have attracted considerable attention due to their promising potential in clinical applications as platforms for drug delivery, biomarkers, and therapeutic tools for diseases.

Single cell analysis is essential to provide a theoretical basis for exploring cellular communication. Electrochemistry is a prominent method that is more frequently applied in molecular determination at the single cell and subcellular level than any other techniques due to its susceptible, label-free, *in situ*, and real time characteristics. The number of molecules released and stored inside single vesicles can also be determined by electrochemistry. A direct correlation between vesicle sizes and release dynamics can be obtained by the combination of electrochemical approaches with different diameters of CNPs. Additionally, quantification of the transmitter concentration in individual vesicles is indirectly achieved correlation of vesicular size and total content.

The main focus of this thesis includes various applications of different electrochemical techniques to study mechanisms of vesicular secretion. Two of the papers discussed present an analytical approach to combine amperometry with open CNPs to characterize vesicular structures and release kinetics to understand the regulation of exocytosis and neurotransmission. These studies have shown the ability to quantify the vesicular content and concentrations of LDCVs and revealed the presence of exosomes-encapsulated chemical messengers.

In the work presented here, only neuroendocrine cells were exploited to monitor catecholamines from vesicles by electrochemical techniques. In the future, the development or modification of new probes would enable one to detect other types of neurotransmitters or non-electroactive molecules. The Huang group has successfully developed an enzymatic biosensor to measure glutamate from hippocampal neurons by co-modification of glutamate oxidase and platinum nanoparticles on the surface of a carbon fiber electrode.[203] Moreover, other cell types such as pancreatic beta cells,[204] human carcinoid BON cells[205] or rat insulinoma INS-1 cells[206] can also be employed to offer more valuable insights into exocytotic process of different biochemical messengers. Therefore, applications of new methodologies together with various biological models will hopefully contribute to open new avenues in the research of molecular biology of chemical neurotransmission.

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References

(1) Keynes, R. D. The Ionic Movements during Nervous Activity. *J Physiol* **1951**, *114*, 119–150.

(2) Hodgkin, A. L. The Ionic Basis of Electrical Activity in Nerve and Muscle. *Biol Rev* **1951**, *26*, 339–409.

(3) Hodgkin, A. L., and Horowicz, P. The influence of potassium and chloride ions on the membrane potential of single muscle fibres. *J Physiol* **1959**, *148*, 127– 160.

(4) Zuber, B.; Nikonenko, I.; Klauser, P.; Muller, D.; Dubochet, J. The Mammalian Central Nervous Synaptic Cleft Contains a High Density of Periodically Organized Complexes. *Proc Natl Acad Sci USA* **2005**, *102*, 19192–19197.

(5) Lembke, A. "Mind" and "Brain". *Am J Psychiatry* **2001**, *158*, 48–59.

(6) Klyachko, V. A.; Jackson, M. B. Capacitance Steps and Fusion Pores of Small and Large-Dense-Core Vesicles in Nerve Terminals. *Nature* **2002**, *418*, 89–92.

(7) Carlsson, A.; Lindqvist, M.; Magnusson, T. 3,4-Dihydroxyphenylalanine and 5-Hydroxytryptophan as Reserpine Antagonists. *Nature* **1957**, *180*, 1200.

(8) Harrington, K. A.; Augood, S. J.; Kingsbury, A. E.; Foster, O. J. F.; Emson, P. C. Dopamine Transporter (DAT) and Synaptic Vesicle Amine Transporter (VMAT2) Gene Expression in the Substantia Nigra of Control and Parkinson's Disease. *Mol Brain Res* **1996**, *36*, 157–162.

(9) Salamone, J. D.; Correa, M. The Mysterious Motivational Functions of Mesolimbic Dopamine. *Neuron* **2012**, *76*, 470–485.

(10) Luciana, M.; Collins, P. F.; Depue, R. A. Opposing Roles for Dopamine and Serotonin in the Modulation of Human Spatial Working Memory Functions. *Cereb Cortex* **1998**, *8*, 218–226.

(11) Fernstrom, J. D.; Fernstrom, M. H. Tyrosine, Phenylalanine, and Catecholamine Synthesis and Function in the Brain. *J Nutr* **2007**, *137*, 1539S-1547S.

(12) Kobayashi, K.; Nagatsu, T. Tyrosine Hydroxylase. *Primer on the Autonomic Nervous System* **2012**, *239*, 45–47.

(13) Blaschko, H. The Activity of l(-)-dopa Decarboxylase. *J Physiol* **1942**, *101*, 337–349.

(14) Christenson, J. G.; Dairman, W.; Udenfriend, S. Preparation and Properties of a Homogeneous Aromatic L-Amino Acid Decarboxylase from Hog Kidney. *Arch Biochem Biophys* **1970**, *141*, 356–367.

(15) Eiden, L. E.; Schäfer, M. K. H.; Weihe, E.; Schütz, B. The Vesicular Amine Transporter Family (SLC18): Amine/Proton Antiporters Required for Vesicular Accumulation and Regulated Exocytotic Secretion of Monoamines and Acetylcholine. *Pflugers Arch - Eur J Physiol* **2004**, *447*, 636–640.

(16) Agnati, L. F.; Zoli, M.; Strömberg, I.; Fuxe, K. Intercellular Communication in the Brain: Wiring versus Volume Transmission. *Neurosci* **1995**, *69*, 711–726.

(17) Liu, C.; Kershberg, L.; Wang, J.; Schneeberger, S.; Kaeser, P. S. Dopamine Secretion Is Mediated by Sparse Active Zone-like Release Sites. *Cell* **2018**, *172*, 706-718.e15.

(18) Kebabian, J. W.; Petzold, G. L.; Greengard, P. Dopamine-Sensitive Adenylate Cyclase in Caudate Nucleus of Rat Brain, and Its Similarity to the "Dopamine Receptor". *Proc Natl Acad Sci USA* **1972**, *69*, 2145–2149.

(19) Miller, G. W.; Gainetdinov, R. R.; Levey, A. I.; Caron, M. G. Dopamine Transporters and Neuronal Injury. *Trends Pharmacol Sci* **1999**, *20*, 424–429.

(20) Berridge, C. W.; Schmeichel, B. E.; España, R. A. Noradrenergic Modulation of Wakefulness/Arousal. *Sleep Med Rev* **2012**, *16*, 187–197.

(21) Cannon, W. B. Bodily changes in pain, hunger, fear, and rage; an account of recent researches into the function of emotional excitement. D Appleton and Company, New York, London **1915**.

(22) Kirshner, N.; Goodall, M. The Formation of Adrenaline from Noradrenaline. *BBA - General Subjects* **1957**, *24*, 658–659.

(23) Brumback, A. C.; Lieber, J. L.; Angleson, J. K.; Betz, W. J. Using FM1-43 to Study Neuropeptide Granule Dynamics and Exocytosis. *Methods* **2004**, *33*, 287–294.

(24) Mansvelder, H. D.; Kits, K. S. Calcium Channels and the Release of Large Dense Core Vesicles from Neuroendocrine Cells: Spatial Organization and Functional Coupling. *Prog Neurobiol* **2000**, *62*, 427–441.

(25) Klingauf, J.; Neher, E. Modeling Buffered Ca2+ Diffusion near the Membrane: Implications for Secretion in Neuroendocrine Cells. *Biophys J* **1997**, *72*, 674–690.

(26) De Camilli, P.; Jahn, R. Pathways to Regulated Exocytosis in Neurons. *Annu Rev Physiol* **1990**, *52*, 625–645.

(27) Bauerfeind, R.; Ohashi, M.; Huttner, W. B. Biogenesis of Secretory Granules and Synaptic Vesicles: Facts and Hypotheses. *Ann NY Acad Sci* **1994**, *733*, 233–244.

(28) Park, Y.; Kim, K. T. Short-Term Plasticity of Small Synaptic Vesicle (SSV) and Large Dense-Core Vesicle (LDCV) Exocytosis. *Cell Signal* **2009**, *21*, 1465–1470.

(29) Kelly, R. B. Storage and Release of Neurotransmitters. *Neuron* **1993**, *10*, 43–53.

(30) Liu, Y.; Schweitzer, E. S.; Nirenberg, M. J.; Pickel, V. M.; Evans, C. J.; Edwards, R. H. Preferential Localization of a Vesicular Monoamine Transporter to Dense Core Vesicles in PC12 Cells. *J Cell Biol* **1994**, *127*, 1419–1433.

(31) Kim, T.; Gondré-Lewis, M. C.; Arnaoutova, I.; Loh, Y. P. Dense-Core Secretory Granule Biogenesis. *Physiol* **2006**, *21*, 124–133.

(32) Crivellato, E.; Nico, B.; Ribatti, D. The Chromaffin Vesicle: Advances in Understanding the Composition of a Versatile, Multifunctional Secretory Organelle. *Anat Rec* **2008**, *291*, 1587–1602.

(33) Albillos, A.; Dernick, G.; Horstmann, H.; Almers, W.; Alvarez de Toledo, G.; Lindau, M. The Exocytotic Event in Chromaffin Cells Revealed by Patch Amperometry. *Nature* **1997**, *389*, 509–512.

(34) Bernard, L. M. Chromogranins and Neurotransmitter Retention During Development of the Adrenergic Neuron. Advances in Behavioral Biology.

(35) Thureson-Klein, Å.; Klein, R. L.; Chen Yen, S. H. Morphological Effects of Osmolarity on Purified Noradrenergic Vesicles. *J Neurocytol* **1975**, *4*, 609–627.

(36) Colliver, T. L.; Pyott, S. J.; Achalabun, M.; Ewing, A. G. VMAT-Mediated Changes in Quantal Size and Vesicular Volume. *J Neurosci* **2000**, *20*, 5276–5282.

(37) Helle, K. B.; Reed, R. K.; Pihl, K. E.; Serck-Hanssen, G. Osmotic Properties of the Chromogranins and Relation to Osmotic Pressure in Catecholamine Storage Granules. *Acta Physiol Scand* **1985**, *123*, 21–33.

(38) Machado, J. D.; Camacho, M.; Alvarez, J.; Borges, R. On the Role of Intravesicular Calcium in the Motion and Exocytosis of Secretory Organelles. *Commun Integr Biol* **2009**, *2*, 71–73.

(39) Fathali, H.; Dunevall, J.; Majdi, S.; Cans, A. S. Extracellular Osmotic Stress Reduces the Vesicle Size While Keeping a Constant Neurotransmitter Concentration. *ACS Chem Neurosci* **2017**, *8*, 368–375.

(40) Ren, L.; Oleinick, A.; Svir, I.; Amatore, C.; Ewing, A. G. Amperometric Measurements and Dynamic Models Reveal a Mechanism for How Zinc Alters Neurotransmitter Release. *Angew Chem Int Ed* **2020**, *59*, 3083–3087.

(41) Taleat, Z.; Estévez-Herrera, J.; Machado, J. D.; Dunevall, J.; Ewing, A. G.; Borges, R. Electrochemical Investigation of the Interaction between Catecholamines and ATP. *Anal Chem* **2018**, *90*, 1601–1607.

(42) Weder, H. G.; Wiegand, U. W. The Interaction of Biogenic Amines with Adenosine-5'-Triphosphate: A Calorimetric Study. *FEBS Lett* **1973**, *38*, 8–10.

(43) Omiatek, D. M.; Dong, Y.; Heien, M. L.; Ewing, A. G. Only a Fraction of Quantal Content Is Released during Exocytosis as Revealed by Electrochemical Cytometry of Secretory Vesicles. *ACS Chem Neurosci* **2010**, *1*, 234–245.

(44) He, X.; Ewing, A. G. Simultaneous Counting of Molecules in the Halo and Dense-Core of Nanovesicles by Regulating Dynamics of Vesicle Opening. *Angew Chem* **2022**, *61*, e202116217.

(45) Rabasco, S.; Nguyen, T. D. K.; Gu, C.; Kurczy, M. E.; Phan, N. T. N.; Ewing, A. G. Localization and Absolute Quantification of Dopamine in Discrete Intravesicular Compartments Using NanoSIMS Imaging. *Int J Mol Sci* **2022**, *23*, 160.

(46) Oleinick, A.; Hu, R.; Ren, B.; Tian, Z.-Q.; Svir, I.; Amatore, C. 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* **2016**, *163*, H3014–H3024.

(47) Matsuo, R.; Yamagishi, M.; Wakiya, K.; Tanaka, Y.; Ito, E. Target Innervation Is Necessary for Neuronal Polyploidization in the Terrestrial Slug Limax. *Dev Neurobiol* **2013**, 609–620.

(48) Ehrlich, M. E.; Evincer, M.; Regunathan, S.; Teitelman, G. Mammalian Adrenal Chromaffin Cells Coexpress the Epinephrine-Synthesizing Enzyme and Neuronal Properties in Vivo and in Vitro. *Dev Biol* **1994**, *163*, 480–490.

(49) Fung, M. M.; Viveros, O. H.; O'Connor, D. T. Diseases of the Adrenal Medulla. *Acta Physiol* **2008**, *192*, 325–335.

(50) Heuser, J. E.; Reese, R.T. Structure of the Synapse. Handbook of Physiology – The Nervous System I, 261–294.

(51) Plattner, H.; Artalejo, A. R.; Neher, E. Ultrastructural Organization of Bovine Chromaffin Cell Cortex - Analysis by Cryofixation and Morphometry of Aspects Pertinent to Exocytosis. *J Cell Biol* **1997**, *139*, 1709–1717.

(52) Jia, X. X; Gorczyca, M.; Budnik, V. Ultrastructure of Neuromuscular Junctions in Drosophila: Comparison of Wild Type and Mutants with Increased Excitability. *J Neurobiol* **1994**, *25*, 893–895.

(53) Wade, P. R.; Westfall, J. A. Ultrastructure of Enterochromaffin Cells and Associated Neural and Vascular Elements in the Mouse Duodenum. *Cell Tissue Res* **1985**, *241*, 557–563.

(54) Moriyama, Y.; Hasuzawa, N.; Nomura, M. Is the Vesicular Nucleotide Transporter a Molecular Target of Eicosapentaenoic Acid? *Front Pharmacol* **2022**, *13*, 1–9.

(55) Cotter, K.; Stransky, L.; McGuire, C.; Forgac, M. Recent Insights into the Structure, Regulation, and Function of the V-ATPases. *Trends Biochem Sci* **2015**, *40*, 611–622.

(56) McGuire, C.; Stransky, L.; Cotter, K.; Forgac, M. Regulation of V-ATPase Activity. *Front Biosci - Landmark* **2017**, *22*, 609–622.

(57) Forgac, M. Vacuolar ATPases: Rotary Proton Pumps in Physiology and Pathophysiology. *Nat Rev Mol Cell Biol* **2007**, *8*, 917–929.

(58) Díaz-Vera, J.; Morales, Y. G.; Hernández-Fernaud, J. R.; Camacho, M.; Montesinos, M. S.; Calegari, F.; Huttner, W. B.; Borges, R.; Machado, J. D. Chromogranin B Gene Ablation Reduces the Catecholamine Cargo and Decelerates Exocytosis in Chromaffin Secretory Vesicles. *J Neurosci* **2010**, *30*, 950–957.

(59) Sawada, K.; Echigo, N.; Juge, N.; Miyaji, T.; Otsuka, M.; Omote, H.; Yamamoto, A.; Moriyama, Y. Identification of a Vesicular Nucleotide Transporter. *Proc Natl Acad Sci USA* **2008**, *105*, 5683–5686.

(60) Varoqui, H.; Erickson, J. D. Vesicular Neurotransmitter Transporters: Potential Sites for the Regulation of Synaptic Function. *Mol Neurobiol* **1997**, *15*, 165–192.

(61) Edwards, R. H. The Neurotransmitter Cycle and Quantal Size. *Neuron* **2007**, *55*, 835–858.

(62) Peter, D.; Jimenez, J.; Liu, Y.; Kim, J.; Edwards, R. H. The Chromaffin Granule and Synaptic Vesicle Amine Transporters Differ in Substrate Recognition and Sensitivity to Inhibitors. *J Biol Chem* **1994**, *269*, 7231–7237.

(63) Peter, D.; Liu, Y.; Sternini, C.; De Giorgio, R.; Brecha, N.; Edwards, R. H. Differential Expression of Two Vesicular Monoamine Transporters. *J Neurosci* **1995**, *15*, 6179–6188.

(64) Südhof, T. C. The Synaptic Vesicle Cycle. *Annu Rev Neurosci* **2004**, *27*, 509–547.

(65) Jahn, R.; Fasshauer, D. Molecular Machines Governing Exocytosis of Synaptic Vesicles. *Nature* **2012**, *490*, 201–207.

(66) Denker, A.; Rizzoli, S. O. Synaptic Vesicle Pools: An Update. *Front Synaptic Neurosci* **2010**, *2*, 1–12.

(67) Burkhardt, P.; Hattendorf, D. A.; Weis, W. I.; Fasshauer, D. Munc18a Controls SNARE Assembly through Its Interaction with the Syntaxin N-Peptide. *EMBO J* **2008**, *27*, 923–933.

(68) Koh, T. W.; Bellen, H. J. Synaptotagmin I, a Ca2+ Sensor for Neurotransmitter Release. *Trends Neurosci* **2003**, *26*, 413–422.

(69) Von Gersdorff, H.; Matthews, G. Inhibition of Endocytosis by Elevated Internal Calcium in a Synaptic Terminal. *Nature* **1994**, *370*, 652–655.

(70) Ceccarelli, B.; Hurlbut, W. P.; Mauro, A. Turnover of Transmitter and Synaptic Vesicles at the Prog Neuromuscular Junction. *J Cell Biol* **1973**, *57*, 499–524.

(71) Mueller, V. J.; Wienisch, M.; Nehring, R. B.; Klingauf, J. Monitoring Clathrin-Mediated Endocytosis during Synaptic Activity. *J Neurosci* **2004**, *24*, 2004–2012.

(72) Fernandez JM; Neher E; Gomperts BD. Capacitance Measurements Reveal Stepwise Fusion Events in Degranulating Mast Cells. *Nature* **1984**, *312*, 453–455.

(73) Artalejo, C. R.; Elhamdani, A.; Palfrey, H. C. Secretion: Dense-Core Vesicles Can Kiss-and-Run Too. *Curr Biol* **1998**, *8*, 62–65.

(74) Fesce, R.; Grohovaz, F.; Valtorta, F.; Meldolesi, J. Neurotransmitter release: fusion or 'kiss-and-run'? *Trends Cell Biol* **1994**, *4*, 1–4.

(75) Mellander, L. J.; Trouillon, R.; Svensson, M. I.; Ewing, A. G. Amperometric Post Spike Feet Reveal Most Exocytosis Is via Extended Kiss-and-Run Fusion. *Sci Rep* **2012**, *2*, 907.

(76) Amatore, C.; Oleinick, A. I.; Svir, I. Reconstruction of Aperture Functions during Full Fusion in Vesicular Exocytosis of Neurotransmitters. *ChemPhysChem* **2010**, *11*, 159–174.

(77) Ren, L.; Mellander, L. J.; Keighron, J.; Cans, A. S.; Kurczy, M. E.; Svir, I.; Oleinick, A.; Amatore, C.; Ewing, A. G. The Evidence for Open and Closed Exocytosis as the Primary Release Mechanism. *Q Rev Biophys* **2016**, *49*, e12.

(78) Majdi, S.; Berglund, E. C.; Dunevall, J.; Oleinick, A. I.; Amatore, C.; Krantz, D. E.; Ewing, A. G. Electrochemical Measurements of Optogenetically Stimulated Quantal Amine Release from Single Nerve Cell Varicosities in Drosophila Larvae. *Angew Chem Int Ed* **2015**, *54*, 13609–13612.

(79) Staal, R. G.; Mosharov, E. V; Sulzer, D. Dopamine Neurons Release Transmitter via a Flickering Fusion Pore. *Nat Neurosci* **2004**, *7*, 341–346.

(80) Phan, N. T. N.; Li, X.; Ewing, A. G. Measuring Synaptic Vesicles Using Cellular Electrochemistry and Nanoscale Molecular Imaging. *Nat Rev Chem* **2017**, *1*, 1–18.

(81) Martin, S. J.; Grimwood, P. D.; Morris, R. G. Synaptic Plasticity and Memory: An Evaluation of The Hypothesis. *Ann Rev Neurosci* **2000**, *23*, 649–711.

(82) Rahamimoff, R.; Fernandez, J. M. Pre- and Postfusion Regulation of Transmitter Release. *Neuron* **1997**, *18*, 17–27.

(83) Chivet, M.; Hemming, F.; Pernet-Gallay, K.; Fraboulet, S.; Sadoul, R. Emerging Role of Neuronal Exosomes in the Central Nervous System. *Front Physiol* **2012**, *3*, 1–6.

(84) Colombo, M.; Raposo, G.; Théry, C. Biogenesis, Secretion, and Intercellular Interactions of Exosomes and Other Extracellular Vesicles. *Annu Rev Cell Dev Biol* **2014**, *30*, 255–289.

(85) Brown, L.; Wolf, J. M.; Prados-Rosales, R.; Casadevall, A. Through the Wall: Extracellular Vesicles in Gram-Positive

Bacteria, Mycobacteria and Fungi. *Nat Rev Microbiol* **2015**, *13*, 620–630.

(86) Raposo, G.; Stoorvogel, W. Extracellular Vesicles: Exosomes, Microvesicles, and Friends. *J Cell Biol* **2013**, *200*, 373–383.

(87) Johnstone, R. M.; Adam, M.; Hammond, J. R.; Orr, L.; Turbide, C. Vesicle Formation during Reticulocyte Maturation. Association of Plasma Membrane Activities with Released Vesicles (Exosomes). *J Biol Chem* **1987**, *262*, 9412–9420.

(88) Jeppesen, D. K.; Fenix, A. M.; Franklin, J. L.; Higginbotham, J. N.; Zhang, Q.; Zimmerman, L. J.; Liebler, D. C.; Ping, J.; Liu, Q.; Evans, R.; et al. Reassessment of Exosome Composition. *Cell* **2019**, *177*, 428-445.e18.

(89) Mathieu, M.; Martin-Jaular, L.; Lavieu, G.; Théry, C. Specificities of Secretion and Uptake of Exosomes and Other Extracellular Vesicles for Cell-to-Cell Communication. *Nat Cell Biol* **2019**, *21*, 9–17.

(90) Buzas, E. I. The Roles of Extracellular Vesicles in the Immune System. *Nat Rev Immunol* **2023**, *23*, 236–250.

(91) Borges, F. T.; Reis, L. A.; Schor, N. Extracellular Vesicles: Structure, Function, and Potential Clinical Uses in Renal Diseases. *Braz J Med Biol Res* **2013**, *46*, 824–830.

(92) Di Vizio, D.; Morello, M.; Dudley, A. C.; Schow, P. W.; Adam, R. M.; Morley, S.; Mulholland, D.; Rotinen, M.; Hager, M. H.; Insabato, L.; et al. Large Oncosomes in Human Prostate Cancer Tissues and in the Circulation of Mice with Metastatic Disease. *Am J Pathol* **2012**, *181*, 1573–1584.

(93) van Niel, G.; Carter, D. R. F.; Clayton, A.; Lambert, D. W.; Raposo, G.; Vader, P. Challenges and Directions in Studying Cell–Cell Communication by Extracellular Vesicles. *Nat Rev Mol Cell Biol* **2022**, *23*, 369–382.

(94) Yipp, B. G.; Petri, B.; Salina, D.; Jenne, C. N.; Scott, B. N. V.; Zbytnuik, L. D.; Pittman, K.; Asaduzzaman, M.; Wu, K.; Meijndert, H. C.; Malawista, S. E.; de Boisfleury Chevance, A.; Zhang, K.; Conly, J.; Kubes, P. Infection-Induced NETosis Is a Dynamic Process Involving Neutrophil Multitasking in Vivo. *Nat Med* **2012**, *18*, 1386–1393.

(95) Ma, L.; Li, Y.; Peng, J.; Wu, D.; Zhao, X.; Cui, Y.; Chen, L.; Yan, X.; Du, Y.; Yu, L. Discovery of the Migrasome, an Organelle Mediating Release of Cytoplasmic Contents during Cell Migration. *Cell Res* **2015**, *25*, 24–38.

(96) Melentijevic, I.; Toth, M. L.; Arnold, M. L.; Guasp, R. J.; Harinath, G.; Nguyen, K. C.; Taub, D.; Parker, J. A.; Neri, C.; Gabel, C. V.; Hall, D. H.; Driscoll, M. C. Elegans Neurons Jettison Protein Aggregates and Mitochondria under Neurotoxic Stress. *Nature* **2017**, *542*, 367–371.

(97) Jaiswal, J. K.; Andrews, N. W.; Simon, S. M. Membrane Proximal Lysosomes Are the Major Vesicles Responsible for Calcium-Dependent Exocytosis in Nonsecretory Cells. *J Cell Biol* **2002**, *159*, 625–635.

(98) Lachenal, G.; Pernet-Gallay, K.; Chivet, M.; Hemming, F. J.; Belly, A.; Bodon, G.; Blot, B.; Haase, G.; Goldberg, Y.; Sadoul, R. Release of Exosomes from Differentiated Neurons and Its Regulation by Synaptic Glutamatergic Activity. *Mol Cell Neurosci* **2011**, *46*, 409–418.

(99) Krämer-Albers, E. M.; Bretz, N.; Tenzer, S.; Winterstein, C.; Möbius, W.; Berger, H.; Nave, K. A.; Schild, H.; Trotter, J. Oligodendrocytes Secrete Exosomes Containing Major Myelin and Stress-Protective Proteins: Trophic Support for Axons? *Proteomics Clin Appl* **2007**, *1*, 1446–1461.

(100) Fauré, J.; Lachenal, G.; Court, M.; Hirrlinger, J.; Chatellard-Causse, C.; Blot, B.; Grange, J.; Schoehn, G.; Goldberg, Y.; Boyer, V.; et al. Exosomes Are Released by Cultured Cortical Neurones. *Mol Cell Neurosci* **2006**, *31*, 642–648.

(101) Emmanouilidou, E.; Melachroinou, K.; Roumeliotis, T.; Garbis, S. D.; Ntzouni, M.; Margaritis, L. H.; Stefanis, L.; Vekrellis, K. Cell-Produced α-Synuclein Is Secreted in a Calcium-Dependent Manner by Exosomes and Impacts Neuronal Survival. *J Neurosci* **2010**, *30*, 6838–6851.

(102) Rajendran, L.; Honsho, M.; Zahn, T. R.; Keller, P.; Geiger, K. D.; Verkade, P.; Simons, K. Alzheimer's Disease β-Amyloid Peptides Are Released in Association with Exosomes. *Proc Natl Acad Sci USA* **2006**, *103*, 11172–11177.

(103) Zöller, M. Tetraspanins: Push and Pull in Suppressing and Promoting Metastasis. *Nat Rev Cancer* **2009**, *9*, 40–55.

(104) Escola, J. M.; Kleijmeer, M. J.; Stoorvogel, W.; Griffith, J. M.; Yoshie, O.; Geuze, H. J. Selective Enrichment of Tetraspan Proteins on the Internal Vesicles of Multivesicular Endosomes and on Exosomes Secreted by Human B-Lymphocytes. *J Biol Chem* **1998**, *273*, 20121–20127.

(105) Morello, M.; Minciacchi, V. R.; Candia, P. De; Yang, J.; Posadas, E.; Kim, H.; Griffiths, D.; Bhowmick, N.; Chung, L. W. K.; Gandellini, P.; Freeman, M. R; Demichelis, F; Di Vizio, D. Large Oncosomes Mediate Intercellular Transfer of Functional MicroRNA. *Cell Cycle* **2013**, *12*, 3526–3536.

(106) Di Vizio, D.; Morello, M.; Dudley, A. C.; Schow, P. W.; Adam, R. M.; Morley, S.; Mulholland, D.; Rotinen, M.; Hager, M. H.; Insabato, L.; et al. Large Oncosomes in Human Prostate Cancer Tissues and in the Circulation of Mice with Metastatic Disease. *Am J Pathol* **2012**, *181*, 1573–1584.

(107) Heijnen, H. F. G.; Schiel, A. E.; Fijnheer, R.; Geuze, H. J.; Sixma, J. J. Activated Platelets Release Two Types of Membrane Vesicles: Microvesicles by Surface Shedding and Exosomes Derived from Exocytosis of Multivesicular Bodies and α-Granules. *Blood* **1999**, *94*, 3791–3799.

(108) Wolf, P. The Nature and Significance of Platelet Products in Human Plasma. *Br J Haematol* **1967**, *13*, 269–288.

(109) Johnstone, R. M.; Mathew, A.; Mason, A. B.; Teng, K. Exosome Formation during Maturation of Mammalian and Avian Reticulocytes: Evidence That Exosome Release Is a Major Route for Externalization of Obsolete Membrane Proteins. *J Cell Physiol* **1991**, *147*, 27–36.

(110) Zaborowski, M. P.; Balaj, L.; Breakefield, X. O.; Lai, C. P. Extracellular Vesicles: Composition, Biological Relevance, and Methods of Study. *Biosci* **2015**, *65*, 783–797.

(111) Colombo, M.; Moita, C.; Van Niel, G.; Kowal, J.; Vigneron, J.; Benaroch, P.; Manel, N.; Moita, L. F.; Théry, C.; Raposo, G. Analysis of ESCRT Functions in Exosome Biogenesis, Composition and Secretion Highlights the Heterogeneity of Extracellular Vesicles. *J Cell Sci* **2013**, *126*, 5553–5565.

(112) Henne, W. M.; Buchkovich, N. J.; Emr, S. D. The ESCRT Pathway. *Dev Cell* **2011**, *21*, 77–91.

(113) Clague, M. J.; Liu, H.; Urbé, S. Governance of Endocytic Trafficking and Signaling by Reversible Ubiquitylation. *Dev Cell* **2012**, *23*, 457–467.

(114) Raiborg, C.; Stenmark, H. The ESCRT Machinery in Endosomal Sorting of Ubiquitylated Membrane Proteins. *Nature* **2009**, *458*, 445–452.

(115) Bonifacino, J. S.; Glick, B. S. The Mechanisms of Vesicle Budding and Fusion. *Cell* **2004**, *116*, 153–166.

(116) Jahn, R.; Scheller, R. H. SNAREs - Engines for Membrane Fusion. *Nat Rev Mol Cell Biol* **2006**, *7*, 631–643.

(117) Kowal, J.; Tkach, M.; Théry, C. Biogenesis and Secretion of Exosomes. *Curr Opin Cell Biol* **2014**, *29*, 116–125.

(118) Bissig, C.; Gruenberg, J. ALIX and the Multivesicular Endosome: ALIX in Wonderland. *Trends Cell Biol* **2014**, *24*, 19–25.

(119) Nabhan, J. F.; Hu, R.; Oh, R. S.; Cohen, S. N.; Lu, Q. Formation and Release of Arrestin Domain-Containing Protein 1-Mediated Microvesicles (ARMMs) at Plasma Membrane by Recruitment of TSG101 Protein. *Proc Natl Acad Sci USA* **2012**, *109*, 4146–4151.

(120) Yang, J. M.; Gould, S. J. The Cis-Acting Signals That Target Proteins to Exosomes and Microvesicles. *Biochem Soc Trans* **2013**, *41*, 277–282.

(121) Shen, B.; Wu, N.; Yang, M.; Gould, S. J. Protein Targeting to Exosomes/Microvesicles by Plasma Membrane Anchors. *J Biol Chem* **2011**, *286*, 14383–14395.

(122) Lässer, C. Exosomes in Diagnostic and Therapeutic Applications: Biomarker, Vaccine and RNA Interference Delivery Vehicle. *Expert Opin Biol Ther* **2015**, *15*, 103–117.

(123) Van Dommelen, S. M.; Vader, P.; Lakhal, S.; Kooijmans, S. A. A.; Van Solinge, W. W.; Wood, M. J. A.; Schiffelers, R. M. Microvesicles and Exosomes: Opportunities for Cell-Derived Membrane Vesicles in Drug Delivery. *J Control Release* **2012**, *161*, 635–644.

(124) Katsuda, T.; Kosaka, N.; Ochiya, T. The Roles of Extracellular Vesicles in Cancer Biology: Toward the Development of Novel Cancer Biomarkers. *Proteomics* **2014**, *14*, 412–425.

(125) D'Souza-Schorey Crislyn, C.; Clancy, J. W. Tumor-Derived Microvesicles: Shedding Light on Novel Microenvironment Modulators and Prospective Cancer Biomarkers. *Genes Dev* **2012**, *26*, 1287–1299.

(126) Amor, S.; Puentes, F.; Baker, D.; Van Der Valk, P. Inflammation in Neurodegenerative Diseases. *Immunology* **2010**, *129*, 154–169.

(127) Albillos, A.; Artalejo, A. R.; López, M. G.; Gandía, L.; García, A. G.; Carbone, E. Calcium Channel Subtypes in Cat Chromaffin Cells. *J Physiol* **1994**, *477*, 197–213.

(128) Cortez, V.; Santana, M.; Marques, A. P.; Mota, A.; Rosmaninho-Salgado, J.; Cavadas, C. Regulation of Catecholamine Release in Human Adrenal Chromaffin Cells by β-Adrenoceptors. *Neurochem Int* **2012**, *60*, 387–393.

(129) Liu, M.; Dunn, P. M.; King, B. F.; Burnstock, G. Rat Chromaffin Cells Lack P2X Receptors While Those of the Guinea-Pig Express a P2X Receptor with Novel Pharmacology. *Br J Pharmacol* **1999**, *128*, 61–68.

(130) Lovell, P. V.; James, D. G.; Mccobb, D. P. Bovine versus Rat Adrenal Chromaffin Cells: Big Differences in BK Potassium Channel Properties. *J Neurophysiol* **2000**, *83*, 3277–3286.

(131) Coupland, R. E. Electron Microscopic Observations on the Structure of the Rat Adrenal Medulla. I. the Ultrastructure and Organization of Chromaffin Cells in the Normal Adrenal Medulla. *J Anat* **1965**, *99*, 231–254.

(132) Montesinos, M. S.; Machado, J. D.; Camacho, M.; Diaz, J.; Morales, Y. G.; De La Rosa, D. A.; Carmona, E.; Castañeyra, A.; Viveros, O. H.; O'Connor, D. T.; Mahata, S. K; Borges, R. The Crucial Role of Chromogranins in Storage and Exocytosis Revealed Using Chromaffin Cells from Chromogranin A Null Mouse. *J Neurosci* **2008**, *28*, 3350–3358.

(133) Yoo, S. H.; Oh, Y. S.; Kang, M. K.; Huh, Y. H.; So, S. H.; Park, H. S.; Park, H. Y. Localization of Three Types of the Inositol 1,4,5-Trisphosphate Receptor/Ca2+ Channel in the Secretory Granules and Coupling with the Ca2+ Storage Proteins Chromogranins A and B. *J Biol Chem* **2001**, *276*, 45806–45812.

(134) Arpino, G.; Wu, L.-G.; Bleck, C. Ultrastructural Preservation and Improved Visualization of Membranes in Primary Bovine Chromaffin Cells. *Leica Microsystem*.

(135) Winkler, H. The Adrenal Chromaffin Granule: A Model for Large Dense Core Vesicles of Endocrine and Nervous Tissue. *J Anat* **1993**, *183*, 237–52.

(136) H Kryvi, T Flatmark, O. T. Comparison of the Ultrastructure of Adrenaline and Noradrenaline Storage Granules of Bovine Adrenal Medulla. *Eur J Cell Biol.* **1979**, *20*, 76–82.

(137) Greene, L. A.; Tischler, A. S. Establishment of a Noradrenergic Clonal Line of Rat Adrenal Pheochromocytoma Cells Which Respond to Nerve Growth Factor. *Proc Natl Acad Sci USA* **1976**, *73*, 2424–2428.

(138) Greene, L. A.; Rein, G. Release, Storage and Uptake of Catecholamines by a Clonal Cell Line of Nerve Growth Factor (NGF) Responsive Pheochromocytoma Cells. *Brain Res* **1977**, *129*, 247–263.

(139) Das, P. C.; McElroy, W. K.; Cooper, R. L. Alteration of Catecholamines in Pheochromocytoma (PC12) Cells in Vitro by the Metabolites of Chlorotriazine Herbicide. *Toxicol Sci* **2001**, *59*, 127–137.

(140) Du, M.; Flanigan, V.; Ma, Y. Simultaneous Determination of Polyamines and Catecholamines in PC-12 Tumor Cell Extracts by Capillary Electrophoresis with Laser-Induced Fluorescence Detection. *Electrophoresis* **2004**, *25*, 1496–1502.

(141) Kumar, G. K.; Overholt, J. L.; Bright, G. R.; Hui, K. Y.; Lu, H.; Gratzl, M.; Prabhakar, N. R. Release of Dopamine and Norepinephrine by Hypoxia from PC-12 Cells. *Am J Physiol Cell Physiol* **1998**, *274*, 1592–1600.

(142) Westerink, R. H. S.; De Groot, A.; Vijverberg, H. P. M. Heterogeneity of Catecholamine-Containing Vesicles in PC12 Cells. *Biochem Biophys Res Commun* **2000**, *270*, 625–630.

(143) Adams, R. D.; Harkins, A. B. PC12 Cells That Lack Synaptotagmin i Exhibit Loss of a Subpool of Small Dense Core Vesicles. *Biophys J* **2014**, *107*, 2838–2849.

(144) Schubert, D.; LaCorbiere, M., Klier, F. G.; Steinbach, J. H. The modulation of neurotransmitter synthesis by steroid hormones and insulin, *Brain Res* **1980**, *190*, 67-79.

(145) Schäfer, T.; Karli, U. O.; Schweizer, F. E.; Burger, M. M. Docking of Chromaffin Granules-A Necessary Step in Exocytosis? *Biosci Rep* **1987**, *7*, 269–279.

(146) Wiatrak, B.; Kubis Kubiak, A.; Piwowar, A.; Barg, E. PC12 Cell Line: Cell Types, Coating of Culture Vessels, Differentiation and Other Culture Conditions. *Cells* **2020**, *9*, 958.

(147) Keshmirian, J.; Bray, G.; Carbonetto, S. The Extracellular Matrix Modulates the Response of PC12 Cells to Nerve Growth Factor: Cell Aggregation versus Neurite Outgrowth on 3-Dimensional Laminin Substrata. *J Neurocytol* **1989**, *18*, 491–504.

(148) Schubert, D.; Heinemann, S.; Kidokoro, Y. Cholinergic Metabolism and Synapse Formation by a Rat Nerve Cell Line. *Proc Natl Acad Sci USA* **1977**, *74*, 2579–2583.

(149) Lombardo, S.; Maskos, U. Role of the Nicotinic Acetylcholine Receptor in Alzheimer's Disease Pathology and Treatment. *Neuropharmacology* **2015**, *96*, 255–262.

(150) Oddo, S.; LaFerla, F. M. The Role of Nicotinic Acetylcholine Receptors in Alzheimer's Disease. *J Physiol Paris* **2006**, *99*, 172–179.

(151) Lin, C.H.; Huang, Y.J.; Lin, C.J.; Lane, H.Y.; Tsai, G. NMDA Neurotransmission Dysfunction in Mild Cognitive Impairment and Alzheimer's Disease. *Curr Pharm Des* **2014**, *20*, 5169–5179.

(152) Italia, M.; Ferrari, E.; Diluca, M.; Gardoni, F. NMDA and AMPA Receptors at Synapses: Novel Targets for Tau and A-Synuclein Proteinopathies. *Biomedicines* **2022**, *10*, 1550.

(153) Peng, X. M.; Tehranian, R.; Dietrich, P.; Stefanis, L.; Perez, R. G. α-Synuclein Activation of Protein Phosphatase 2A Reduces Tyrosine Hydroxylase Phosphorylation in Dopaminergic Cells. *J Cell Sci* **2005**, *118*, 3523–3530.

(154) Perez, R. G.; Waymire, J. C.; Lin, E.; Liu, J. J.; Guo, F.; Zigmond, M. J. Erratum. A Role for α-Synuclein in the Regulation of Dopamine Biosynthesis. *J Neurosci* **2002**, *22*, 3090–3099.

(155) Castillo, J.; Katz, B. Quantal Components of the End-Plate Potential. *Transplant Proc* **1954**, *124*, 560–573.

(156) Fatt, P.; Katz, B. Spontaneous Subthreshold Activity at Motor Nerve Endings. **1952**, *117*, 109–128.

(157) Martin, A. R. Quantal Nature of Synaptic Transmission. *Physiol Rev* **1966**, *46*, 51.

(158) Neher, E.; Marty, A. Discrete Changes of Cell Membrane Capacitance Observed under Conditions of Enhanced Secretion in Bovine Adrenal Chromaffin Cells. *Proc Natl Acad Sci USA* **1982**, *79*, 6712–6716.

(159) Leszczyszyn, D. J.; Jankowski, J. A.; Viveros, O. H.; Diliberto, E. J.; Near, J. A.; Wightman, R. M. Nicotinic Receptor-Mediated Catecholamine Secretion from Individual Chromaffin Cells: Chemical Evidence for Exocytosis. *J Biol Chem* **1990**, *265*, 14736–14737.

(160) Wightman, R. M.; Jankowski, J. A.; Kennedy, R. T.; Kawagoe, K. T.; Schroeder, T. J.; Leszczyszyn, D. J.; Near, J. A.; Diliberto, E. J.; Viveros, O. H. Temporally Resolved Catecholamine Spikes Correspond to Single Vesicle Release from Individual Chromaffin Cells. *Proc Natl Acad Sci USA* **1991**, *88*, 10754–10758.

(161) Chow, R. H.; Ruden, L. von; Neher, E. Delay in Vesicle Fusion Revealed by Electrochemical Monitoring of Single Secretory Events in Adrenal Chromaffin Cells. *Nature* **1992**, *356*, 60–63.

(162) Zhou, Z.; Misler, S.; Chow, R. H. Rapid Fluctuations in Transmitter Release from Single Vesicles in Bovine Adrenal Chromaffin Cells. *J Biophys* **1996**, *70*, 1543–1552.

(163) Dunevall, J.; Fathali, H.; Najafinobar, N.; Lovric, J.; Wigstrom, J.; Cans, A. S.; Ewing, A. G. Characterizing the Catecholamine Content of Single Mammalian Vesicles by Collision-Adsorption Events at an Electrode. *J Am Chem Soc* **2015**, *137*, 4344–4346.

(164) Li, X.; Majdi, S.; Dunevall, J.; Fathali, H.; Ewing, A. G. Quantitative Measurement of Transmitters in Individual Vesicles in the Cytoplasm of Single Cells with Nanotip Electrodes. *Angew Chem Int Ed* **2015**, *54*, 11978–11982.

(165) Marcenac, F.; Gonon, F. Fast in Vivo Monitoring of Dopamine Release in the Rat Brain with Differential Pulse Amperometry. *Anal Chem* **1985**, *57*, 1778–1779.

(166) Kissinger, P. T.; Hart, J. B.; Adams, R. N. Voltammetry in Brain Tissue - a New Neurophysiological Measurement. *Brain Res* **1973**, *55*, 209–213.

(167) Leszczyszyn, D. J.; Jankowski, J. A.; Viveros, O. H.; Diliberto, E. J.; Near, J. A.; Wightman, R. M. Nicotinic Receptor-Mediated Catecholamine Secretion from Individual Chromaffin Cells: Chemical Evidence for Exocytosis. *J Biol Chem* **1990**, *265*, 14736–14737.

(168) Garguilo, M. G.; Huynh, N.; Proctor, A.; Michael, A. C. Amperometric Sensors for Peroxide, Choline, and Acetylcholine Based on Electron Transfer between Horseradish Peroxidase and a Redox Polymer. *Anal Chem* **1993**, *65*, 523–528.

(169) Hafez, I.; Kisler, K.; Berberian, K.; Dernick, G.; Valero, V.; Yong, M. G.; Craighead, H. G.; Lindau, M. Electrochemical Imaging of Fusion Pore Openings by Electrochemical Detector Arrays. *Proc Natl Acad Sci USA* **2005**, *102*, 13879–13884.

(170) Lin, Y.; Trouillon, R.; Svensson, M. I.; Keighron, J. D.; Cans, A. S.; Ewing, A. G. Carbon-Ring Microelectrode Arrays for Electrochemical Imaging of Single Cell Exocytosis: Fabrication and Characterization. *Anal Chem* **2012**, *84*, 2949–2954.

(171) Zhang, B.; Adams, K. L.; Luber, S. J.; Eves, D. J.; Heien, M. L.; Ewing, A. G. Spatially and Temporally Resolved Single-Cell Exocytosis Utilizing Individually Addressable Carbon Microelectrode Arrays. *Anal Chem* **2008**, *80*, 1394–1400.

(172) Keighron, J. D.; Wang, Y.; Cans, A.-S. Electrochemistry of Single-Vesicle Events. *Annu Rev Anal Chem* **2020**, *13*, 159–181.

(173) Majdi, S.; Berglund, E. C.; Dunevall, J.; Oleinick, A. I.; Amatore, C.; Krantz, D. E.; Ewing, A. G. Electrochemical Measurements of Optogenetically Stimulated Quantal Amine Release from Single Nerve Cell Varicosities in Drosophila Larvae. *Angew Chem Int Ed* **2015**, *54*, 13609–13612.

(174) Pothos, E. N.; Davila, V.; Sulzer, D. Presynaptic Recording of Quanta from Midbrain Dopamine Neurons and Modulation of the Quantal Size. *J Neurosci* **1998**, *18*, 4106–4118.

(175) Zhou, Z.; Misler, S. Amperometric Detection of Stimulus-Induced Quantal Release of Catecholamines from Cultured Superior Cervical Ganglion Neurons. *Proc Natl Acad Sci USA* **1995**, *92*, 6938–6942.

(176) Li, Y. T.; Zhang, S. H.; Wang, L.; Xiao, R. R.; Liu, W.; Zhang, X. W.; Zhou, Z.; Amatore, C.; Huang, W. H. Nanoelectrode for Amperometric Monitoring of Individual Vesicular Exocytosis inside Single Synapses. *Angew Chem Int Ed* **2014**, *53*, 12456–12460.

(177) Lv, J.; Wang, X. Y.; Chang, S.; Xi, C. Y.; Wu, X.; Chen, B. Bin; Guo, Z. Q.; Li, D. W.; Qian, R. C. Amperometric Identification of Single Exosomes and Their Dopamine Contents Secreted by Living Cells. *Anal Chem* **2023**, *95*, 11273–11279.

(178) Trouillon, R.; Ewing, A. G. Single Cell Amperometry Reveals Glycocalyx Hinders the Release of Neurotransmitters during Exocytosis. *Anal Chem* **2013**, *85*, 4822–4828.

(179) Trouillon, R.; Ewing, A. G. Actin Controls the Vesicular Fraction of Dopamine Released during Extended Kiss and Run Exocytosis. *ACS Chem Biol* **2014**, *9*, 812–820.

(180) Taleat, Z.; Larsson, A.; Ewing, A. G. Anticancer Drug Tamoxifen Affects Catecholamine Transmitter Release and Storage from Single Cells. *ACS Chem Neurosci* **2019**, *10*, 2060–2069.

(181) Ren, L.; Oleinick, A.; Svir, I.; Amatore, C.; Ewing, A. G. Amperometric Measurements and Dynamic Models Reveal a Mechanism for How Zinc Alters Neurotransmitter Release. *Angew Chem Int Ed* **2020**, *59*, 3083–3087.

(182) Omiatek, D. M.; Santillo, M. F.; Heien, M. L.; Ewing, A. G. Hybrid Capillary-Microfluidic Device for the Separation, Lysis, and Electrochemical Detection of Vesicles. *Anal Chem* **2009**, *81*, 2294–2302.

(183) Omiatek, D. M.; Dong, Y.; Heien, M. L.; Ewing, A. G. Only a Fraction of Quantal Content Is Released during Exocytosis as Revealed by Electrochemical Cytometry of Secretory Vesicles. *ACS Chem Neurosci* **2010**, *1*, 234–245.

(184) Omiatek, D. M.; Bressler, A. J.; Cans, A. S.; Andrews, A. M.; Heien, M. L.; Ewing, A. G. The Real Catecholamine Content of Secretory Vesicles in the CNS Revealed by Electrochemical Cytometry. *Sci Rep* **2013**, *3*, 1447.

(185) Dunevall, J.; Fathali, H.; Najafinobar, N.; Lovric, J.; Wigstrom, J.; Cans, A. S.; Ewing, A. G. Characterizing the Catecholamine Content of Single Mammalian Vesicles by Collision-Adsorption Events at an Electrode. *J Am Chem Soc* **2015**, *137*, 4344–4346.

(186) Li, X.; Majdi, S.; Dunevall, J.; Fathali, H.; Ewing, A. G. Quantitative Measurement of Transmitters in Individual

Vesicles in the Cytoplasm of Single Cells with Nanotip Electrodes. *Angew Chem Int Ed* **2015**, *54*, 11978–11982.

(187) Li, X.; Ren, L.; Dunevall, J.; Ye, D.; White, H. S.; Edwards, M. A.; Ewing, A. G. Nanopore Opening at Flat and Nanotip Conical Electrodes during Vesicle Impact Electrochemical Cytometry. *ACS Nano* **2018**, *12*, 3010–3019.

(188) Singhal, R.; Bhattacharyya, S.; Orynbayeva, Z.; Vitol, E.; Friedman, G.; Gogotsi, Y. Small Diameter Carbon Nanopipettes. *Nanotechnology* **2010**, *21*, 015304.

(189) Kim, B. M.; Murray, T.; Bau, H. H. The Fabrication of Integrated Carbon Pipes with Sub-Micron Diameters. *Nanotechnology* **2005**, *16*, 1317.

(190) Shao, Z.; Puthongkham, P.; Hu, K. K.; Jia, R.; Mirkin, M. V.; Venton, B. J. Thin Layer Cell Behavior of CNT Yarn and Cavity Carbon Nanopipette Electrodes: Effect on Catecholamine Detection. *Electrochim Acta* **2020**, *361*, 137032.

(191) Schrlau, M. G.; Brailoiu, E.; Patel, S.; Gogotsi, Y.; Dun, N. J.; Bau, H. H. Carbon Nanopipettes Characterize Calcium Release Pathways in Breast Cancer Cells. *Nanotechnology* **2008**, *19*, 325102.

(192) Clarke, R. W.; White, S. S.; Zhou, D.; Ying, L.; Klenerman, D. Trapping of Proteins under Physiological Conditions in a Nanopipette. *Angew Chem Int Ed* **2005**, *44*, 3747–3750.

(193) Levis, R. A.; Rae, J. L. The Use of Quartz Patch Pipettes for Low Noise Single Channel Recording. *J Biophys* **1993**, *65*, 1666–1677.

(194) Munoz, J.-L.; Coles, J. A. Quartz Micropipettes for Intracellular Voltage Microelectrodes and Ion-Selective Microelectrodes. *J Neurosci Methods* **1987**, *22*, 57–64.

(195) Hu, K.; Wang, D.; Zhou, M.; Bae, J. H.; Yu, Y.; Xin, H.; Mirkin, M. V. Ultrasensitive Detection of Dopamine with Carbon Nanopipets. *Anal Chem* **2019**, *91*, 12935–12941.

(196) Pan, R.; Hu, K.; Jiang, D.; Samuni, U.; Mirkin, M. V. Electrochemical Resistive-Pulse Sensing. *J Am Chem Soc* **2019**, *141*, 19555–19559.

(197) Zhu, W. Y.; Gu, C. Y.; Dunevall, J.; Ren, L.; Zhou, X. M.; Ewing, A. G. Combined Amperometry and Electrochemical Cytometry Reveal Differential Effects of Cocaine and Methylphenidate on Exocytosis and the Fraction of Chemical Release. *Angew Chem Int Ed* **2019**, *58*, 4238–4242.

(198) Castaing, R.; Slodzian, G. Optique Corpusculaire-Premiers Essais de Microanalyse Par Emission Ionique Secondaire. *J Microscopie* **1962**, *1*, 395–410.

(199) Sigmund, P. Sputtering by Ion Bombardment: Theoretical Concepts Introductory and Historical Survey. In *Topics in Applied Physics* **1981**, *47*, 9–71.

(200) Benninghoven, Alfred.; Sichtermann, W. K. Detection, Identification, and Structural Investigation of Biologically Important Compounds by Secondary Ion Mass Spectrometry. *Anal Chem* **1978**, *50*, 1180–1184.

(201) Edmond, D. H.; Stroobant, V. Mass Spectrometry: Principles and Applications. *John Wiley & Sons* **2007**.

(202) Mamyrin, B. A.; Karataev, V. I.; Shmikk, D. V.; Zagulin, V. A. The Mass-Reflectron, a New Nonmagnetic Time-of-Flight Mass Spectrometer with High Resolution. *Zh Eksp Teor Fiz* **1973**, *64*, 82–89.

(203) Yang, X. K.; Zhang, F. L.; Wu, W. T.; Tang, Y.; Yan, J.; Liu, Y. L.; Amatore, C.; Huang, W. H. Quantitative Nano-Amperometric Measurement of Intravesicular Glutamate Content and Its Sub-Quantal Release by Living Neurons. *Angew Chem Int Ed* **2021**, *60*, 15803–15808.

(204) Braun, M.; Ramracheya, R.; Johnson, P. R.; Rorsman, P. Exocytotic Properties of Human Pancreatic β-Cells. *Ann N Y Acad Sci* **2009**; *1152*, 187–193.

(205) Wang, Y.; Gu, C.; Patel, B. A.; Ewing, A. G. Nano-Analysis Reveals High Fraction of Serotonin Release during Exocytosis from a Gut Epithelium Model Cell. *Angew Chem Int Ed* **2021**, *60*, 23552–23556.

(206) Xiong, X.; Zhou, K. M.; Wu, Z. X.; Xu, T. Silence of Synaptotagmin I in INS-1 Cells Inhibits Fast Exocytosis and Fast Endocytosis. *Biochem Biophys Res Commun* **2006**, *347*, 76–82.