The role of presynaptic proteins in maintaining neuronal viability

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General introduction

From brain to synapses

The human brain is a complex organ responsible for the regulation of many processes in the body: motor control, thinking, learning and memory, perception, judgment and other cognitive functions. In order to receive, integrate and process information, a complex network of neurons communicate with each other. The human brain is composed by of an estimated 100 billion neurons and probably even 10 times more supporting glial cells. In higher organisms, the nervous system uses signaling circuits composed of many neurons. These circuits run through the distinct areas of the brain: the cerebrum, the diencephalon, the cerebellum and the brainstem. The cerebrum is the outer layer and the largest region of the brain and is important for memory, language, consciousness, etc. The cerebrum surrounds the diencephalon, which includes the hypothalamus, the pituitary gland and the thalamus and is responsible for endocrine functions, motor functions, homeostasis, directing sense impulses throughout the body, etc. The diencephalon together with the subcortical and cortical area of the brain forms the limbic system. The hippocampus is located under the cerebral cortex and is part of the cortical areas that form the limbic system. It is important for consolidation of memory, short-term memory to long-term memory and is well known for spatial memory studies in rodents. The cerebellum is located below the cerebrum and posterior to the brainstem. It receives sensory inputs important for body movements, posture and balance. It is also important for controlling fine movements such as writing and talking. The brainstem is the most inferior structure of the brain and connects the brain to the spinal cord. The spinal cord and the brain together form the central nervous system (CNS) while the peripheral nervous system (PNS) contains the nerves and ganglia outside of the brain and the spinal cord. The PNS connects the limbs and organs to the CNS by the autonomous nervous system and the somatic nervous system. The somatic nervous system can be further divided into sensory nervous system or somatosensory system. The dorsal-root ganglion (DRG) contains the cell bodies of sensory neurons that carry information from the periphery, e.g. the muscles, to the brain, especially to two populations of neurons localized at the spinal cord, interneurons and motor neurons. These neurons are, unlike most other neurons, pseudo-unipolar and have one axon with two branches: central and peripheral[1].

The communication between neurons occurs through a typically long axonal projection that conducts electrical signals (action potentials), away from the cell body of a neuron, to the dendrite projections of a receiving neuron or to other target cells (muscle cells, glands, etc.). Neurons are electrical polarized cells in which, the resting membrane potential is a result of a net negative charge in the interior of the cell. Membrane depolarization opens the voltage-gated ion channels leading to an inward flow of sodium ions. Action potentials are all or none and move at a speed up to 100m/s. After a brief recovery period, neurons can fire repeatedly, up to several hundred hertz. During the action potential, voltage-gated calcium channels in the axon nerve terminal open and calcium ion influx stimulates the release of chemical neurotransmitters. Following the action potential, sodium channels close and the potassium channels are then activated leading to an outward current of potassium ions that restores the negative-inside resting potential[1].

Synapses are specialized junctions where communication between a presynaptic and a postsynaptic neuron occurs. In the human brain, 100 trillion of synapses provide the computational capability required for the human body functions. There are two different types of synapses: electrical and chemical synapses. The electrical synapses depend on gap junction channels that link a presynaptic neuron to a postsynaptic neuron allowing the electric information to travel almost instantaneous. In electrical synapses the presynaptic cell does not need to reach a threshold at which it causes an action potential. Instead, the electrical current continues into the next cell and causes depolarization in proportion to the current. In chemical synapses the pre- and postsynaptic neurons are separated by a synaptic cleft of 20nm. When an action potential arrives at the chemical synapse, neurotransmitters (e.g. glutamate or acetylcholine) are released from the presynaptic neuron. In the synaptic cleft, neurotransmitters diffuse from the site of release and bind to specific receptors at the postsynaptic neurons, leading to depolarization of the postsynaptic membrane. A chemical synapse is slower than an electrical synapse, taking around 0.5 to 5ms for a signal to cross the synapse[1].

At the postsynaptic membrane, a dense protein network contains the neurotransmitter receptors, together with scaffold molecules, signaling enzymes and cytoskeletal components. Many synaptic neurotransmitter receptors are ionotropic receptors (ligand-gated channels) for glutamate and γ-aminobutyric acid (GABA), which determine, together with the type of neurotransmitter released, if the synapse is excitatory or inhibitory. The organization of excitatory and inhibitory postsynaptic sites is different in morphology and molecular composition. Excitatory synapses typically have a postsynaptic density, visible as an electron-dense structure in electron micrographs that contain many proteins implicated in psychiatric illness[2].

At the presynaptic terminal, the cytomatrix proteins, Bassoon, Piccolo and others, make a structural framework together with cytoskeletal proteins. The active zone is the presynaptic region where synaptic vesicles and fusion machinery accumulate to orchestrate fast and precise synaptic transmission[3]. RIM proteins are central elements in this process together with specific binding partners such as RIM-BPs, Rab3 and Rab27, Munc13 and Ca^{2+} channels[3]. The neuronal soluble NSF attachment protein receptor (SNARE) proteins that drive synaptic vesicle fusion in synapses consist of the target or t-SNAREs (syntaxin-1 and synaptosomal-associated protein of 25 kDa protein (SNAP-25)) and vesicular or v-SNAREs (synaptobrevin-2/VAMP2)[4,5]. These three proteins assemble into the SNARE complex by forming a four helical bundle that promotes fusion assisted by other presynaptic proteins such as Munc18-1[6] (Fig. 1.1). The zippering of the four SNARE motifs, syntaxin-1, SNAP-25, VAMP2 and Munc18-1, leads to the fusion of synaptic vesicles with the plasma membrane together with other proteins, including the Ca^{2+} sensor synaptotagmin-1 and complexins[6]. Hence, the recruitment and assembly of this molecular machinery and the release of neurotransmitters are regulated in a tight manner. Failures in the proper functioning in this complex regulatory network of proteins have been demonstrated in several neurodegenerative diseases and mental disorders.

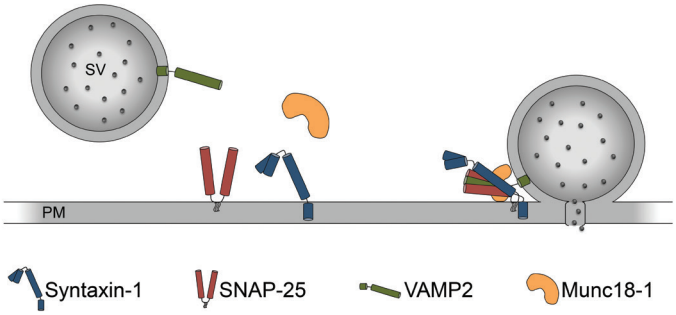

Figure 1.1 - Schematic representation of the SNARE complex. Presynaptic proteins are crucial for exocytosis of synaptic vesicles with release of neurotransmitters followed by endocytosis and synaptic vesicle recycling (for a review see[7]). The zippering of the four SNARE motifs, syntaxin-1 (blue), SNAP-25 (red), Synaptobrevin-2/VAMP2 (green) and the sec1/Munc18-like (SM) protein (Munc18-1 (orange)), leads to the fusion of synaptic vesicles (SV) with the plasma membrane (PM). Adapted from[8,9].

Neurodegenerative diseases

Neurodegenerative diseases affect millions of people worldwide and occur when nerve cells in the CNS or peripheral nervous system lose their ability to function and eventually die. This will affect body movement and coordination (known as ataxia) and mental functioning (for instance dementia). Alzheimer's disease (AD), Parkinson's disease (PD), Amyotrophic lateral sclerosis (ALS), Huntington's disease and Spinal muscular atrophy are among the most prevalent neurodegenerative diseases, with AD and PD as the most common. In addition to age, a combination of genes and environmental factors increases the risk of developing a neurodegenerative disease. Although some treatments might improve the symptoms, relieve the pain and increase mobility, there is currently no cure or ways of delaying the progression of neurodegenerative diseases.

AD is characterized by progressive impairment of memory, a short-term memory in early stages, cognitive disabilities, personality changes and culminating in a complete dependence on others. The most important risk factor for AD is aging and the most important genetic susceptibility factor is the Apolipoprotein E (ApoE) gene. Rare mutations in other genes, such as presenilins and amyloid precursor protein (APP) cause a rare familial form of AD[10]. The hallmarks of AD are the extracellular plaques formed by the aggregation of amyloid beta (Aβ) peptides, which are typically surrounded by neurons with dystrophic neurites and the neurofibrillary tangles (NFTs), intracellular filamentous aggregates of hyperphosphorylated tau[10]. APP processing leads to the formation of Aβ peptides. In the amyloidogenic pathway, the sequential cleavage of APP by β-secretase (beta-site amyloid precursor protein cleaving enzyme 1 (BACE1)) and γ-secretase releases the soluble extracellular domain of APP (sAPPβ), Aβ peptide and the intracellular carboxy-terminal domain of APP (AICD)[10-12]. From the Aβ peptides formed, Aβ42 is more prone to aggregation and believed to be more neurotoxic than Aβ40[10]. In the anti-amyloidogenic pathway the cleavage of APP by α-secretase prevents Aβ formation, producing sAPPα, p3 fraction and AICD[10,13].

Changes in synaptic stability and connectivity are often believed to precede the

loss of neurons and play a relevant role in a broad range of neurodegenerative diseases[14]. In AD, synaptic failure was initially observed in studies that showed a reduction in synapse density[15]. Even at a late stage of the disease, synaptic loss seems to be more robustly correlated with cognitive deficit than the number of plaques or tangles[15]. For instance, expression of a presynaptic vesicle protein, synaptophysin, was found to correlate with the cognitive decline in AD patients[16,17]. Consistent with this, it was observed in AD mouse models that the reduction of synaptophysin occurs well before Aβ plaque formation. Finally, the decrease in synapses correlates with an increase of soluble Aβ, before Aβ plaques are formed[18-20]. Understanding the role of these early alterations in AD is an important step to fully understand the mechanism of this neurodegenerative disease and to design early intervention strategies.

Presynaptic proteins crucial for synaptic function were found to be altered in AD and PD patients. In AD postmortem brain tissue, a decrease in VAMP2 and Munc18-1 was reported together with an increase in syntaxin-1 levels[21]. Another study showed an increase in Munc18-1 levels in AD brain tissue[22], while a third study showed no changes in the levels of the SNARE proteins: syntaxin-1, SNAP-25 and VAMP2 in postmortem brain tissue from AD and PD patients[23]. Thus, although changes are reported in presynaptic proteins, these results are not unanimous among different studies.

Another link between Munc18-1 and AD is the fact that Munc18-1 has been suggested to bind APP[24]. Furthermore, Munc18-1 and its interaction partner Mint were shown to be important for the spatial separation of APP and BACE1[24]. In resting neurons, APP associates with syntaxin-1 microdomains through Munc18-1 and Mint[24]. When Munc18-1 is phosphorylated by cdk5, a shift from APP-syntaxin-1 to APP-BACE1 clusters is observed[24]. Thus, it was suggested that Munc18-1 phosphorylation state, together with its binding to syntaxin-1, might be important for the mechanism underlying APP-BACE1 spatial separation (Fig. 1.2).

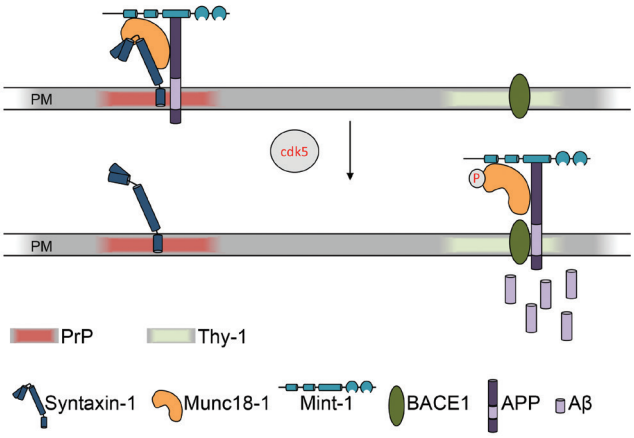

Figure 1.2 - Illustration of APP and BACE spatial separation in presence of Munc18-1. Munc18-1, syntaxin-1 and Mint-1 anchor APP in the PrP microdomain. Upon cdk5 stimulation, modification in Munc18-1/syntaxin-1 complex are observed shifting APP to the Thy-1 microdomain to associate with BACE1. This shift results in an increase of Aβ production.

Apart from their role in neurodegenerative diseases, some presynaptic proteins have been shown to be crucial to maintain neuronal survival *in vitro* such as syntaxin-1, SNAP-25 and Munc18-1[25-29], while other proteins such as VAMPs or Munc13-1/2 do not lead to cell loss in culture[30,31] (discussed below). However, it is currently not known which cell death mechanisms are triggered by depletion of presynaptic proteins.

Cell death as normal aspect of brain development

Brain development starts with differentiation of neuronal precursor cells into a complex neuronal network[32]. Upon birth, neurons gradually stop dividing, although the brain development continues with the maturation of synapses and neurons[32]. Throughout this process several of these neurons and synapses are eliminated via a highly regulated process known as programmed cell death (PCD)[33,34]. Although in the past, PCD has been used as synonym of apoptosis, it is now known, that non-apoptotic mechanisms of PCD also exist[35,36]. Thus, different forms of PCD have been described: type I – apoptosis, type II - autophagy and type III - cytoplasmic or regulated necrosis (necroptosis)[35,36]. Furthermore, cell death can also occur via a mechanism that does not exactly fit in any of these major classes[35,36]. Although cell death is part of the normal brain development, aberrant neuronal cell loss occurs in neurodegenerative diseases. However, it is still unknown which molecular mechanisms of cell death are involved.

Mechanisms of neuronal cell death

Molecular mechanisms of cell death – apoptosis, necrosis and/or autophagy – are involved in neurodegenerative diseases. These mechanisms are trigged by cellular stress, which varies in nature and duration. Different signaling pathways can affect the fate of apoptosis, therefore it is important to understand which signaling pathways are involved in neurodegeneration.

Apoptosis

Apoptosis is a programmed suicide mechanism that is often presented by membrane blebbing, cell shrinkage and chromatin condensation[37]. Furthermore, mitochondria membrane permeabilization[38] and Golgi fragmentation[39] have been described as hallmarks of apoptosis. Apoptosis is activated by the intrinsic, also known as mitochondrial, or the extrinsic, also known as death-receptor, pathways.

The intrinsic pathway is controlled and activated by the Bcl-2 family of proteins [40-42]. Activation of pro-apoptotic factors will disrupt the mitochondrial outer membrane, releasing cytochrome c that will then activate the cysteinyl aspartate protease (caspase) 9[40-42]. The extrinsic pathway requires activation of the cell surface death receptors, such as tumor necrosis receptors (TNF) superfamily (as TNFR, FAS, TRAIL) leading to caspase activation by FAS-associated death domain protein (FADD) and TNFR-associated death domain TRADD[43-46]. As an end step, these two pathways activate caspase 3, caspase 6 and caspase 7[47-50]. Furthermore, different signaling pathways such as Mitogen-activated protein kinases/c-Jun N-terminal kinases (MAPK/JNKs), nuclear factor-κB (NF-κB), c-Myc and phosphoinositide 3-kinase (PI3K)/protein kinase B (PKB, also known as Akt) (PI3K/Akt) pathway can affect apoptosis.

The PI3K/Akt pathway can be activated by extracellular signaling by neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor I (IGFI) and glial cell line-derived neurotrophic factor (GDNF), through the dimerization of tropomyosin receptor kinase (Trk)[51-53]. Upon this dimerization, PI3K is activated and phosphatidylinositol 4,5-biphosphate (PI(4,5)P2) is converted into phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P3)[54] (Box 1). Effector proteins, such as Akt and its activator phosphoinositide-dependent protein kinase-1 (PDK-1), translocate to the cell membrane where they bind to PI(3,4,5)P3, via the pleckstrin-homology (PH) domain and undergo phosphorylation/activation[55-57]. Akt is activated by phosphorylation of PDK1 at threonine 308 and by the mammalian target of rapamycin (mTOR) and DNA-dependent protein kinase (DNA-PK) at serine 473[58-60]. Akt phosphorylation attenuates the activation of the intrinsic apoptotic pathway by inhibiting forkhead (FOX) proteins that induce the expression of pro-apoptotic factors; by activating X-linked inhibitor of apoptosis protein (XIAP), a protein that inhibits apoptosis; or by regulating the expression of anti-apoptotic genes by activating the transcription factor cyclic AMP response element-binding protein (CREB) and the IκB kinase (IKK), a positive regulator of NF-κB[61,62]. A parallel way to activate PI3K is through the Ras G-protein, insulin receptor substrate (IRS), and Gab-1 that directly activates Trk and therefore stimulates PI3K activation[63,64]. In summary, cell death can occur via apoptosis, a controlled/programmed cell death mechanism, by several interconnected pathways.

Box 1. Phosphoinositides

Phosphoinositides (PIs) are the phosphorylated products of phosphatidylinositol and are essential components of eukaryotic cell membranes, being crucial for organelle physiology, vesicular trafficking, cell growth and survival[65,66]. Phosphoinositides correspond to less than 10% of the total of phospholipids in animal cells and have a fast turnover in the brain[67]. Phosphatidylinositol is a glycerophospholipid and consists of an inositol ring, linked via a phosphodiester bond, to a diacylglycerol (DAG) (two non-polar fatty acids tails covalently bound to a glycerol) backbone[65,68,69]. Phosphorylation of phosphatidylinositol occurs in one of the five hydroxyl (-OH) groups of the inositol ring and leads to seven different PIs[69]. These seven different species of PIs have different cell functions and are localized at specific cell compartments[65] (Fig. 1.3).

PI(4)P is one of the most abundant PIs, mainly present at the Golgi but also found at endosomal compartments and plasma membrane[70]. Perturbation in PI(4)P levels by phosphatidylinositol 4-kinases (PI4K) and suppressor of actin mutations 1 (Sac1) phosphatase affects the structure and the function of the Golgi[71].

PI(4)P generates PI(4,5)P2 by phosphorylation of type I phosphatidylinositol phosphate kinases (PIPKIs) at position 5[54]. PI(4,5)P2 has been implicated as signaling molecule that regulates various cell processes such as vesicular trafficking, endocytosis, actin cytoskeleton, channel and transporter activities and signal transduction as a precursor of intracellular

messengers generated by phospholipases[65,69]. Furthermore, PI(4,5)P2 has also been linked to apoptosis[72-74 72-75], as well as to exocytosis by sequestering presynaptic proteins crucial for this process[76].

PI(4,5)P2 is converted into PI(3,4,5)P3 by PI3K[62,77] and the phosphatase and tensin homolog (PTEN) converts it back to PI(4,5)P2[78]. PI(3,4,5)P3 is localized mainly at the PM, concentrated in lipid rafts[79]. In resting cells, the levels of this phosphoinositide are low, however the concentration can increase in response to growth factors[79]. PI(3,4,5)P3 is important for cell proliferation and differentiation, metabolic changes and survival[62,80,81].

Biochemistry and microscopy techniques where developed to specifically detect and manipulate individual phosphoinositides, since changes in levels and localization have been linked to cell dysregulation. For biochemical detection techniques, different methods can be applied: 1) radioactivity methods - metabolic labeling ([^{3}H] inositol or [^{32}P] organic phosphate) and thin layer chromatography (TLC) or high-performance liquid chromatography (HPLC) separation of deacylated PIs; 2) non-radioactivity methods - HPLC separation of deacylated PIs followed by suppressed conductivity measurements and mass spectrometry (MS)[82,83]. Radioactivity methods allow detection of PIs subspecies while the non-radioactive HPLC methods are less sensitive and do not detect all PIs subspecies[82,83]. Combining MS with HPLC improves sensitive and specificity without using radiolabeling[82]. Biochemical detection techniques have the limitation of measuring PI levels at a specific time point and therefore the dynamic changes of PIs are not characterized. An additional caveat to consider is that the spatial separation and PIs distribution is not measured[82,83]. On the other hand, these methods provide the advantage of measuring different PIs simultaneously, allowing the detection of compensatory PI changes[82,83].

Microscopy detection techniques measure PIs relative levels and localization by using probes of specific protein domains fused to fluorescent tags or antibodies[82]. Antibody-based PIs detection allows detection of PIs by immunohistochemistry. This is a semi-quantitative method that depends on sample fixation (and therefore lipid extraction by fixatives or detergents can occur), does not allow the detection of transient PIs changes and proteins bound to PIs might not deliver accurate results[82]. The use of protein domains for PIs detection has increased the knowledge of PIs biology, since it allows measuring PIs in live cells, however not without some caveats. By overexpressing these probes in the cell, PIs interaction with other proteins can be disrupted and therefore downstream cell signaling pathways can be altered. Another caveat is the fact that some of these protein domains recognize more than one subspecies of PIs[82]. Manipulation techniques allow changes of PIs levels by 1) genetically or chemically perturbing PI metabolizing enzymes; 2) application of PI analogs; 3) inducible acute manipulations (electrogenetic-, chemical-genetic and optogenetic manipulations)[82]. The use of microscopy detection techniques allows a more detailed study of PIs function in live cells.

Although the available techniques allow the study of PIs levels and localization, some caveats exist making these techniques not as good as detection techniques for proteins. Improving the available techniques is crucial for more precise PIs results. Furthermore, combining existing

live cell imaging techniques with super resolution microscopy would provide higher spatial and temporal resolution in real time.

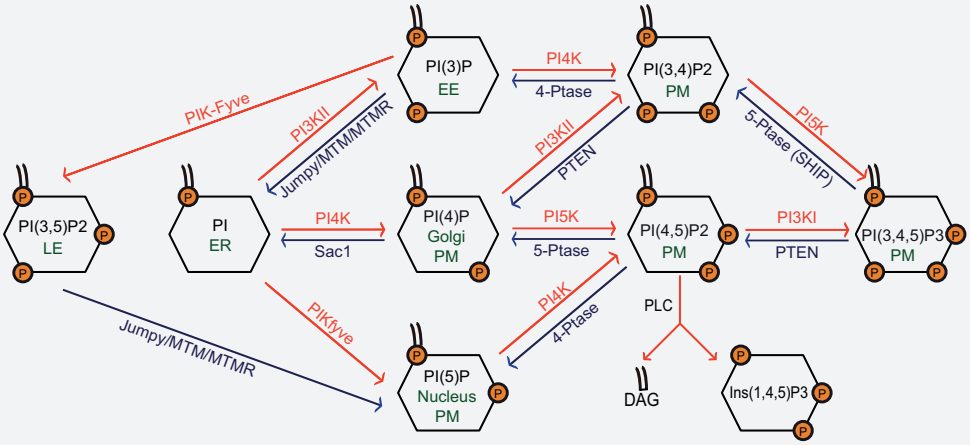

Figure 1.3 – Schematic representation of the Phosphoinositides metabolism. *Kinases are shown in red, phosphatases in blue. EE - early endosome; LE – Late endosome; ER – endoplasmic reticulum; PM – plasma membrane. Adapted from*[68,84].

Necrosis

Necrosis was first described as an unregulated mode of cell death, caused by injury or disease. However, it is now accepted that necrosis can occur in a highly regulated/programmed manner, suggesting that cellular signaling pathways initiate a necrosis response - necroptosis[36,85]. Although necroptosis refers to only one type of regulated necrosis, the term is now used as a synonym. Necrosis is characterized by cell swelling, loss of cell membrane integrity and release of cell products into the extracellular space[86]. Contrasting with the fragmentation of the nucleus in apoptotic cells, necrotic cells show a distended and largely intact nucleus[87]. The release of intracellular products results in the activation of several receptors[88,89] and in the initiation of an inflammatory response[90].

Necroptosis refers to a non-apoptotic mode of cell death. One way of inducing necroptosis is triggered by the ligation of TNF-receptor 1 when caspases are inhibited[36]. Receptor-interacting serine/threonine protein kinase 1 (RIPK1) and RIPK3 are crucial kinases in TNF-induced regulated necrosis[85]. During this process a RIPK1 and TRADD (TNFR1-associated death domain)-dependent receptor-bound complex I is formed[36,85]. Polyubiquitination of RIPK1 influences the transition from complex I to cytosolic death-inducing signaling complex (DISC)[87]. Complex I activates NF-κB leading to upregulation of anti-apoptotic genes[36,85]. Furthermore, necroptosis can also be induced by other components, such as FAS (TNFRSF6 or CD95), poly-(ADP ribose) polymerase-1 (PARP-1), toll-like receptor (TLR), DNA-dependent activator of interferon-regulatory factors (DAI) and protein kinase R (PKR)[36,85]. Although apoptosis has been linked to various neurodegenerative diseases, in the last years, regulated necrosis has also been shown as a prominent mode of cell death.

Autophagy

The link between autophagy and cell death was not always well established. For many years autophagy was described only as a dynamic cellular pathway, critical for cell homeostasis, protein turnover and clearance through lysosome degradation[91,92]. Autophagy was only later accepted as a form of PCD. During brain development an increase in autophagy was observed in combination with cell death[91]. Furthermore, impaired autophagy can result in an increase of damaged organelles triggering other cell death mechanisms - apoptosis or necrosis[91]. Autophagic cells are characterized by double-membrane vesicles – autophagosomes - where proteins and organelles are sequestered[86,93].

The mTOR kinase is the major regulator of autophagy. Its activation occurs by inhibiting the tuberous sclerosis complex 1 (TCS1) and TCS2 leading to the activation of GTP-binding protein Rheb. This inhibition is controlled by insulin or growth factors by activating the PI3K/Akt pathway and inhibition of AMP kinase (AMPK)[91]. Furthermore, activation of autophagy genes (Atg)-related proteins coordinate the specific steps in autophagy[91]. Autophagy can also be activated by an mTOR-independent signaling pathway[94].

Neurons are more susceptive to dysfunctional autophagy than mitotic cells, due to the asymmetric inheritance of degraded proteins during cell division in mitotic cells[95,96] and to the specific neuronal structures: axons and dendrites, which require high-energy demand and protein turnover. In addition, the long axons require a well-regulated transport of autophagosomes from synapses through the axons to the lysosomes at the soma[91,94].

According to the intensity and duration of the stimulus, apoptosis, necrosis and autophagy can occur simultaneous in the cell[38]. Understanding the mechanisms of cell death, as well as the specific pathway(s) involved in neurodegeneration, might pinpoint, in the affected pathway(s), new targets for treatment.

Neuronal cell death in neurodegenerative diseases

Neurodegenerative diseases occur when neuronal viability is not preserved leading to neuronal cell death via different mechanisms of cell death: apoptosis, necrosis and/or autophagy. AD is characterized by extracellular accumulation of Aβ and intercellular neurofibrillary tangles[97]. Deposition of α-synuclein - Lewy bodies in dopaminergic neurons are hallmarks of Parkinson's disease PD[98]. Although these neurodegenerative diseases have independent triggers the same cell death processes can be activated.

Necrotic cells have been detected in AD postmortem human brain tissue[99,100]. Increased apoptotic markers are also observed in AD patients compared to age matched controls[101-103]. In AD, apoptosis occurs mainly via the intrinsic pathway, with activation of caspases[104,105]. However, the role of caspases in AD is not only as executers of apoptosis, but also in upstream processes, such as caspase-mediated proteolysis APP, with an increase of Aβ peptide[106,107]. Conversely, caspases can be activated by Aβ peptide[108]. NTFs were first described in association with caspases in areas where cell death occurred and recently the involvement of caspases in NTFs formation has also been shown[109,110].

Autophagy plays a dual role – pathogenic *vs.* beneficial – in AD. Defective autophagy has

been implicated in the pathogenesis of AD by promoting neuronal cell death, while a protective effect has been associated with the degradation of Aβ and tau[91,92]. Brains of AD patients show an increase in immature autophagic vacuoles (AVs) inside dystrophic neurites, suggesting defects in AVs maturation and retrograde traffic to somatic lysosomes[111]. Furthermore, intracellular Aβ inclusions have been shown in AVs[112] and inhibition of the mTOR pathway in AD transgenic mouse models reduces Aβ and tau pathology by increasing autophagy[113,114]. Thus, autophagy dysregulation in combination with defective clearance of AVs containing Aβ, results in Aβ agglomeration and enhancing of neuronal cell death. In addition, autophagy has been linked to AD progression. In an early stage AVs are increased due to defective organelles and APP stress[94], while in a later AD stage, the degradation and maturation of autophagosomes is blocked as well as the fusion with lysosomes[94].

Similar to AD, also in PD more than one cell death process can be observed, apoptosis, necrosis and/or autophagy[115]. Thus, although a clear link exists between apoptosis/autophagy/necrosis and neurodegenerative diseases, different cell death mechanisms are involved in the different stages of the disease. Furthermore, neurodegeneration has been reported in a large number of studies for genes not involved in the pathways discussed above. One example is kinesin family member 1A (KIF1A), where degeneration occurs via an additional unknown function of KIF1A[116]. Similarly, depletion of presynaptic proteins from neurons leads to neurodegeneration via an unexplained mechanism.

The Golgi apparatus and neurodegenerative diseases

In 1898, Camilo Golgi, using light microscopy, discovered the Golgi apparatus[117]. A mammalian Golgi apparatus consists of cisternae, flattened membrane-enclosed sacs (Golgi stacks) and associated vesicles[118]. The Golgi apparatus is responsible for modification and sorting of proteins. Proteins coming from the ER-Golgi intermediate compartment enter the Golgi apparatus via the *cis*-Golgi[118]. Proteins are further transported to the Golgi stack (divided into sub-compartments, medial and trans) where the majority of protein modifications take place. Lastly, the proteins enter the *trans*-Golgi network that acts as a sorting and distribution center, targeting proteins to the lysosomes, the plasma membrane or to the exterior of the cell[118,119]. In neurons, the Golgi apparatus is localized at the soma with some discontinuous structures - Golgi outposts - localized at the neurites, which facilitate the local secretory traffic[120].

The Golgi apparatus is a very dynamic organelle where different models have been proposed: 1) anterograde vesicular transport between stable compartments by COPI and COPII-vesicles that carry proteins between the cisternae of the Golgi[121]; 2) cisternal progression/maturation, proteins are carried through the Golgi in Golgi cisternae that gradually maturate and move to the *trans*-Golgi[121]; 3) cisternal progression/maturation with heterotypic tubular transport, an extension of the model two where a heterotypic tubular connection, that links the mammalian Golgi "vertically", allows fast anterograde traffic of secretory cargoes and/or retrograde traffic of resident Golgi proteins[121]; 4) rapid partitioning in a mixed Golgi, suggesting the Golgi as a single compartment with processing and exporting domains[121].

Intracellular membrane trafficking requires several SNARE complexes, responsible for

anterograde and retrograde transport between the endoplasmic reticulum (ER) and the Golgi. In mammals, the SNARE complex responsible for the transport from the ER to the ER–Golgi intermediate compartment (ERGIC) is comprised by the following proteins: syntaxin-5/GS27/Sec22b/Bet1 with Sly1 as the corresponding SM-(munc18-like) protein[122]. Between the ERGIC and the *cis*-Golgi the SNARE complex includes syntaxin-5/GS28/Ykt6 that bind to the cognate v-SNARE Bet1 assisted by the SM protein Sly1[122,123]. The same SM protein and t-SNAREs, but a different v-SNARE, GS15, are responsible for the transport between TGN and *cis*-Golgi[122]. Retrograde transport between the *cis*-Golgi and the ER requires the formation of a complex that comprises syntaxin-18/Sec20/Slt1/Sec22b, with the SM protein currently not known[122,124]. These SNARE proteins, responsible for the intracellular membrane trafficking, are isoforms of the SNARE proteins that work at the synapses: syntaxin-1, SNAP-25 and VAMP2 and the SM protein Munc18-1. To what extent these proteins are unique for the steps mentioned here and to what extent the same mechanisms work in all cells of the body and during all phases of development is currently not known.

Two types of abnormal Golgi morphology have been described: condensed and fragmented. The condensed or compacted Golgi apparatus was first observed as a consequence of depolymerization of F-actin by actin toxins such as Latrunculin B (LatB), Cytochalasin D (CytoD) and Clostridium Botulinum C2 toxin (C2 toxin)[125,126]. A recent model explains the condensed Golgi by disrupting the bridge between the Golgi and the cytoskeleton[127]. The Golgi apparatus has a high concentration of phosphatidylinositol 4-phosphate (PI(4)P)[65]. Thus, in this model, the bridge between the Golgi and the cytoskeleton is made by GOLPH3 that binds to PI(4)P at the Golgi and Myosin18A (MYO18A) at the cytoskeleton, since MYO18A binds to F-actin[127]. Disrupting these interactions by knocking down one of these two proteins leads to a condensed Golgi[127,128].

The Golgi apparatus also interacts with the minus ends of microtubules. Golgi structure and position in the cell depend on the microtubule cytoskeleton and molecular motors[129]. Depolymerization of microtubules by nocodazole leads to a different type of abnormal morphology, a fragmented Golgi and a dispersion of Golgi ministacks throughout the cell at ER exit sites[129]. Inhibition of dynein, a tubulin motor protein, also leads to a fragmented Golgi[129-131]. Different reasons have been suggested: 1) the disruption of the binding between dynein and dynactin, since dynactin binds Arp1 that binds to βIII spectrin at the Golgi membrane and depletion of this protein produces a fragmented Golgi[129]; 2) the discovery that Golgin160 is a dynein adaptor provided a new explanation for how dynein maintains the Golgi structure[129]. Golgi disassembly can also be induced by brefeldin A (BFA), a fugal metabolite that blocks intracellular transport by preventing association of COP-I coat to intra-Golgi transport vesicles and enhancing Golgi tubulation. These tubules fuse with the ER, leading to disappearance of a distinct Golgi[132,133].

During mitosis, the Golgi apparatus undergoes normal and reversible disassembly/fragmentation, producing clusters of vesicles that disperse through the cytoplasm[39,134]. An irreversible Golgi fragmentation occurs during apoptosis, in part due to caspase-mediated cleavage of Golgi proteins[39,135,136]. Dysregulation of vesicular traffic[137,138] and increased neuronal

activity can lead to a fragmented Golgi[139]. This process can be reversible by returning neuronal activity to resting levels[139]. Additionally, Golgi fragmentation can occur by breaking down Golgi membranes into smaller vesicles or by fusion with ER and emerging as ER sites, after recycling, dispersed in the cytoplasm[120].

In neurodegenerative diseases, loss of neuronal cell bodies and axons is preceded by an irreversible Golgi fragmentation. A fragmented Golgi was described in ALS motor neurons, where it was compared to the Golgi fragmentation induced by depolymerization of microtubules[140,141]. It was later shown that Golgi fragmentation is an early event, prior to apoptosis[140,142,143]. Golgi structure relies on normal bidirectional transport with the ER[144]. In ALS Golgi-associated vesicular traffic is inhibited as well as ER-Golgi traffic[120]. Dysregulation of vesicular traffic can lead to ER stress and accumulation of proteins at the Golgi that can originate a fragmented Golgi[145,146].

A fragmented Golgi has also been described in AD patients[147,148]. This fragmentation might be caused by modification of structural Golgi proteins[149,150]. Increase in Aβ levels activates cdk5 (cyclin-dependent kinase 5) that will then phosphorylate Golgi structural proteins such as GM130 and Golgi Reassembly Stacking Protein 65 (GRASP65) inducing Golgi fragmentation and enhancing APP trafficking[149,150]. While a fragmented Golgi enhances protein transport, a condensed Golgi reduces protein traffic[127,150]. Furthermore, a fragmented Golgi has also been described in postmortem human brain from PD patients[151].

In summary, an interconnection between defects in Golgi morphology and defects in the secretory pathway can affect proteins essential for neuronal functions. Furthermore, since Golgi defects appear earlier than cell death defects, the Golgi apparatus might be a potential target for drug development in neurodegenerative diseases.

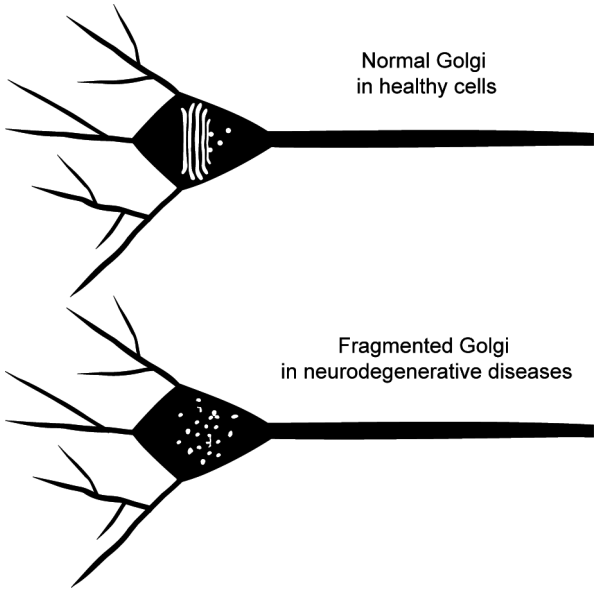

Figure 1.4 - Illustration of different Golgi morphology in neurons. A normal Golgi morphology in health neurons and a fragmented Golgi observed in neurodegenerative diseases' neurons. Adapted from[120].

Presynaptic proteins and neurodegeneration

Presynaptic proteins have been shown to be dysregulated in neurodegenerative diseases such as AD and PD[21-23] and to play a role in neuronal survival[25,26]. In table 1, a detailed list of presynaptic proteins and their impact on neuronal survival is presented. Among presynaptic proteins involved in exocytosis, depletion of t-SNAREs (syntaxin-1/SNAP-25) and SM (Munc18-1) proteins leads to neuronal cell loss[25-29], while neurons depleted of v-SNAREs (VAMPs) do not degenerate in culture[30]. Furthermore, depletion of Munc13-1/2 leads to synaptically silent neurons, without signs of degeneration[31].

Depletion of endocytic proteins, such as endophilin and synaptojanin-1, leads to neurodegeneration at a later stage *in vivo*, while *in vitro* no neuronal cell death is observed[152,153]. For dynamins, a residual low level of neuronal dynamin-2 is sufficient to prevent neuronal cell death in cultured neurons from dynamin-1/-3 double knockout (DKO) mice[154].

In summary, depletion of endocytic proteins leads to a short life span and degeneration in mice, while depletion of exocytic proteins leads to embryonic lethality. Furthermore, *in vitro* neuronal degeneration is observed for t-SNAREs and Munc18-1 but not for v-SNAREs. Currently, it is not known which cell death mechanisms are triggered by depletion of t-SNAREs, SM and endocytic proteins.

Munc18-1

Munc18-1 was first discovered in rat brain as a binding partner of syntaxin-1[155-157]. In mammals, two non-neuronal isoforms of Munc18-1 (or Munc18a) were identified, Munc18-2 (or Munc18b) and Munc18-3 (or Munc18c), with 62% and 51% homology, respectively[158]. Munc18-1 presents 65% homology with *Drosophila melanogaster* (*D. melanogaster*) Ras opposite (Rop), 59% with *Caenorhabditis elegans* (*C. elegans*) unc-18 and 27% with *Saccharomyces cerevisiae* (*S. cerevisiae*) sec-1[157].

Munc18-1 interaction with the SNARE complex is essential for synaptic transmission[159,160] (Fig. 1.2). Furthermore, in neurons lacking Munc18-1 synaptic transmission is abolished[27]. This function is conserved from yeast to mammals. Yeast sec-1 mutants show a blocked secretory pathway with accumulated vesicles unable to fuse[161]; unc-18 *null* nematode worms show a total paralyses[162] and in *D. melanogaster*, Rop is required for synaptic transmission[163,164]. Furthermore, Munc18-1 plays a role in vesicle docking[165-168] and priming by promoting SNARE complex-mediated membrane fusion[169-173].

Munc18-1 and syntaxin-1 are co-chaperones, based on a decrease in syntaxin-1 levels in Munc18-1 KO as well as a decrease in Munc18-1 levels in syntaxin-1A KO brain[160,166,174]. Munc18-1 also regulates syntaxin-1 targeting to plasma membrane in heterologous cells[175-178]. However, in neurons, absence of Munc18-1 does not affect syntaxin-1 targeting to synapses[174]. The binding between these two presynaptic proteins occurs through two sites: 1) via the H_{abc} of syntaxin-1, when syntaxin-1 is in its closed conformation, through Munc18-1 domain 2, preventing the formation of the SNARE complex[155,179-181]; 2) via syntaxin-1 N-peptide, when syntaxin-1 is in its open conformation, exposing the SNARE motif, allowing syntaxin-1 to participate in the SNARE complex formation[159,182,183].

Mouse models	*Embryonic lethality*	*Life span of mice*	*Degeneration in cultured neurons*	*Other cells*
Munc18-1 KO	lethal[27]	-----	die within DIV3-7[26,27]	DRGs survive[184]
SNAP-25 KO	lethal[25]	-----	2% survives till DIV14[25,28]	----
Syntaxin-1A KO	not lethal[185]	die within 12months[185]	survive[185,186]	----
Syntaxin-1B KO	not lethal[186]	die between P7-P14[186]	die between DIV7-9 20% survives[186]	----
Syntaxin-1A/B DKO	lethal[29,187]	-----	few neurons survive till DIV14-21 (from E12.5)[187] neurons die after DIV4 (from E14)[29]	----
VAMP2 KO	lethal[30]	-----	survive[30]	----
TI-VAMP/VAMP7 KO	not lethal[188,189]	normal life span[188,189]	survive[188,189]	----
Munc13-1/2 DKO	lethal[31]	-----	survive[31]	----
Synaptotagmin-1 KO	not lethal[190]	die within 46h[190]	survive[190]	----
Complexin-1 KO	not lethal[191]	die within 2-4months[191]	survive[191]	----
Complexin-2 KO	not lethal[191]	die after 2months[191]	survive[191]	----
Complexin-1/2 DKO	not lethal[191]	die within a few hours[191]	survive[191]	----
CSPα KO	not lethal[192]	die between 2w - 3m[192]	survive[192,193]	----
CAPS1 KO	not lethal[194]	die within 10-30min[194]	survive[194]	----
CAPS2 KO	not lethal[195]	normal life span[195]	survive[195]	----
CAPS1/2 DKO	lethal[195]	-----	survive[195]	----
Synaptojanin-1 KO	not lethal[153]	85% die within 24h rest within 2weeks[153]	survive[153]	----
Dynamin-1 KO	not lethal[196]	die within 2weeks[196]	survive[196]	----
Dynamin-2 KO	lethal[197]	-----	----	Fibroblasts survive (from cKO)[154]
Dynamin-3 KO	not lethal[154]	normal life span[154]	survive[154]	----
Dynamin-1/3 DKO	not lethal[154]	die within several hours[154]	survive[154]	----
Dynamin-1/2/3 cTKO	----	----	----	Fibroblasts survive (stop proliferate ~ DIV4)[198]
Endophilin-1/2/3 TKO	not lethal[152]	die immediately or within a few hours[152]	survive[152]	----

Table 1.1 – Overview of degeneration caused by depletion of presynaptic proteins in vivo and in vitro.

Sec1 temperature-sensitive mutants show deficiency in cell growth and cell death starts after 4 hours at 37°C[161], while unc-18 mutants present slow growth and small body size[199]. Likewise, rop mutant cells are unable to proliferate normally and cell death occurs within the first few divisions[164].

In mice, absence of Munc18-1 is lethal upon birth, nevertheless the brain is assembled properly and synapses are formed, but no maturation is observed[27,200]. In coronal brain sections from Munc18-1 KO mice, neurodegeneration is observed from embryonic day 14 (E14) onwards. The neurodegeneration observed occurs after initial synaptogenesis when neurons undergo massive apoptosis[27]. In culture, Munc18-1 KO neurons degenerate leading to cell death between 3 to 7 days in vitro (DIV)[26]. Trophic support with insulin or BDNF, as well as co-culturing with wild-type neurons, delays but cannot prevent Munc18-1 KO cell loss[26]. In CNS, cell-specific depletion of Munc18-1 results in neurodegeneration of Munc18-1 deficient neurons[26]. However, in primary sensory neurons, no degeneration is observed upon depletion of Munc18-1[184], suggesting a cell intrinsic mechanism specific to CNS neurons. Furthermore, in induced neurons (iN) derived from human H1 embryonic stem (ES) cells where the *munc18-1* gene has been inactivated, neurons degenerate after 1 week and massive cell death is observed during 3 weeks in culture[201].

Syntaxin-1

Syntaxin-1 was originally discovered as a marker for the CNS in adult rat retina[202]. A later study showed the presence of two very similar proteins, syntaxin-1A and syntaxin-1B, at the plasma membrane of synaptic sites[203]. This protein plays a role in docking of synaptic vesicles at the active zone and is essential for exocytosis[203,204].

Syntaxin-1 expression starts in early embryonic development (E10) and its levels are upregulated during synapse formation and brain maturation[205-207]. Syntaxin homologues in yeast, *S. cerevisiae* Sso1 and Sso2 mutants show a post-Golgi transport accumulation[208]. Syntaxin-1A is essential for calcium-regulated secretion in PC12 cells[209] and cleavage of syntaxin-1 by Botulinum Neurotoxin C (BoNT/C) leads to a decrease in vesicle fusion[204]. In *D. melanogaster* syx-1A mutants evoked neurotransmitter release is blocked[210,211].

Syntaxin-1A is essential for embryonic viability and development of *D. melanogaster*[210]. In mammals, syntaxin-1A and syntaxin-1B have distinct roles in survival [186]. While syntaxin-1A KO mice are viable for at least 12 months and fertile[185,212], syntaxin-1B KO mice are born alive, but die within 14 days[186]. Syntaxin-1A is not necessary for normal brain and synapse development or survival in cultured neurons[185,186]. Without syntaxin-1B, brain development is hampered and cultured neurons die around DIV 9 with 20% of the neurons surviving till DIV 14 with normal morphology[186]. Syntaxin-1A/B DKO animals are embryonic lethal. Only a few cultured neurons from E12.5 survive between DIV 14-21[187] and cultured neurons from E14 animals die around DIV 4[29].

SNAP-25

SNAP-25 is essential for synaptic vesicle release and depletion of this protein leads to a reduced spontaneous release and arrest of evoked release[25,28]. SNAP-25 is expressed at low levels in embryonic brain, increasing its expression until adulthood[213]. Subcellular expression of this protein changes over development, from the axons and cell bodies to presynaptic terminals[213]. SNAP-25 is crucial for spontaneous neurotransmitter release and evoked synaptic transmission

is completely abolished in SNAP-25 KO[25]. Furthermore, a reduction in synaptic vesicle endocytosis is observed in absence of SNAP-25[28,214,215].

The major brain structures of SNAP-25 KO animals, such as hippocampus and neocortex are morphologically normal; no neuronal loss is observed, neither changes in other presynaptic proteins[25]. In the absence of SNAP-25, synapses are formed but cultured neurons degenerate after DIV 7[25,28] and between DIV 10-14 only 2% of SNAP-25 KO neurons remain in culture[28]. Overexpression of different splice variants of SNAP-25 (SNAP25a and SNAP25b)[216] or a non-neuronal ubiquitously expressed homologue, SNAP-23[217] rescues survival of SNAP-25 KO neurons[28].

Neurotoxins

The importance of SNARE proteins for neurotransmission and survival became apparent in studies using Botulinum Neurotoxins (BoNTs) - seven different serotypes (BoNT/A to BoNT/G) - produced by the bacterium *Clostridium botulinum* and Tetanus Neurotoxin (TeNT) produced by *Clostridium tetani*[218]. SNARE proteins are cleaved by specific BoNTs, syntaxin-1 is cleaved by BoNT/C, SNAP-25 is cleaved by BoNT/A, BoNT/C and BoNT/E and VAMP2 is cleaved by TeNT, BoNT/B, BoNT/D, BoNT/F and BoNT/G[218]. Application of BoNT/C to neurons leads to widespread neuronal apoptosis within 36h-48h and inhibition of neurite growth[219-221]. BoNT/C does not cleave syntaxin-2/-3/-4, these syntaxin-1 isoforms are able to rescue survival of cultured neurons treated with BoNT/C[221]. In contrast, neurons treated with TeNT do not degenerate in culture[222,223]. Thus, in line with genetic depletion of these presynaptic proteins, proteolytic cleavage, by neurotoxins, of t-SNAREs but not v-SNAREs leads to neuronal cell death.

Despite the profound effects on cell viability, the cell death mechanism(s) in neurons lacking t-SNAREs (syntaxin-1 or SNAP-25) and Munc18-1 are still unknown. Understanding which degenerative mechanism(s) explain cell loss and identifying common or shared mechanism(s) might provide new links between synaptic defects and neurodegenerative diseases.

Thesis Outline

The general aim of this thesis is to investigate the molecular mechanism(s) of neuronal cell death triggered by the absence of specific presynaptic proteins, as well as the role of these proteins in neurodegenerative diseases such as AD. In **chapter 2** we set out to systematically compare the cell death mechanism(s) in mutant mouse models lacking presynaptic proteins. We have shown that depletion of t-SNAREs and Munc18-1 leads to neurodegeneration, while depletion of v-SNAREs does not lead to degeneration despite the synaptic defects. We have further investigated the neuronal cell death mechanisms by studying cell organelles in mutant mouse models that degenerate in culture and have identified a *cis*-Golgi defect in Munc18-1 KO and SNAP-25 KO neurons.

Phosphoinositides, specifically PI(4)P and actin depolymerization, play an important role in Golgi morphology. Therefore in **chapter 3** we have further investigated the *cis*-Golgi defect observed in Munc18-1 KO neurons. We have studied changes in PI(4)P levels

and localization, in actin depolymerization linked to Golgi dynamics and overexpressed Golgi Phosphoprotein 3 (GOLPH3) and vacuolar protein sorting 45 (VPS45), of which the knockdown/knockout phenotypes resemble the *cis*-Golgi in Munc18-1 KO neurons. Our data exclude VPS45-, GOLPH3- and phosphatidylinositol 4-kinase IIIα (PI4KIIIα)-dysfunction as single causes of the *cis*-Golgi abnormalities in Munc18-1 KO neurons. However, we have found a decrease in PI(4)P levels at the Golgi and its kinase, PI4KIIIα, suggesting that changes in phosphoinositide metabolism might contribute to the condensed *cis*-Golgi in Munc18-1 KO neurons.

In **chapter 4**, we further studied phosphoinositides and their role in neuronal cell death. Our data show that the decrease in PI(4,5)P2 levels is not causal to Munc18-1 KO cell loss, since phosphatidylinositol 4-phosphate 5-kinase (PIP5K) overexpression, to increase PI(4,5)P2 levels, did not restore neuronal survival. Furthermore, we investigated the PI3K/Akt pathway and observed an increase in PI(3,4,5)P3 and a decrease in the activation of Akt, suggesting that Munc18-1 KO cell death might occur via this pathway.

Munc18-1 has been suggested to play a role in the spatial separation of APP and BACE1 and to interact with APP[24]. In **chapter 5**, we have further characterized this interaction and showed that absence of Munc18-1 leads to an increase in APP levels in mouse brain. Furthermore, we describe an interaction between Munc18-1 and BACE1, a crucial enzyme that cleaves APP in AD.

To finalize, **chapter 6** summarizes the main findings described in this thesis and provides a broader discussion in relation to the existing literature. The first part focuses on the role of presynaptic proteins in neurodegeneration and the possible links with phosphoinositides, Golgi and actin dynamics. In the second part we discuss our findings in the context of neurodegenerative diseases.