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Jenny Johansson

Karolinska Institutet

From the Rolf Luft Research Center for Diabetes and Endocrinology, Department of Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden

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The essence of all is communication

ABSTRACT

The process by which cells release substances through fusion of vesicles with the plasma membrane is called exocytosis. Regulated exocytosis needs to be tightly controlled in order to respond to the large variation in stimuli and demands for release of neurotransmitters, peptides and hormones. Disturbances in release underlie a multitude of neuropsychiatric disorders and hormonal imbalances. This thesis aimed at exploring exocytotic regulatory mechanisms, focusing on protein isoforms and phosphorylation.

Central for exocytosis is the formation of a trans-membrane SNARE protein complex, consisting of VAMP2, Syntaxin 1 and SNAP-25. The *in vivo* importance of two SNAP-25 isoforms was investigated in a transgenic mouse model in which SNAP-25b expression was replaced with supplementary SNAP-25a, thereby retaining physiological levels of SNAP-25. SNAP-25a is expressed early in development whereas SNAP-25b is the dominant isoform in adult brain. The SNAP-25b null mutants exist in two versions, unfloxed with a *neo* cassette retained and reduced levels of SNAP-25 expression, and floxed with the selection gene excised. Both models demonstrated reduced short-term plasticity in the hippocampus and developed seizures with accompanying changes in neuropeptide expression. Unfloxed mouse mutants had a more severe phenotype with developmental defects and early lethality. Adult floxed mouse mutants show cognitive impairments with increased anxiety and deficits in spatial learning and with time, morphological changes in the hippocampus appeared.

Cdk5 was initially identified as a kinase important for neurite outgrowth but is emerging as a regulator of cell signaling. For activity Cdk5 is dependent on its activator proteins, p35 and p39. We investigated the roles of Cdk5 and the activators in synaptic vesicle and secretory granule exocytosis. In the neuroblastoma-glioma cell line NG108-15, Cdk5 and p35 proteins were preferentially distributed to soluble pools. p39 was expressed at low levels and primarily present in membrane and cytoskeletal fractions. Recordings of postsynaptic membrane potentials in co-cultures with NG108-15 cells and differentiating muscle cells demonstrated that a dominant negative Cdk5 mutant inhibited spontaneous transmitter release. Overexpression of either Cdk5 activator p35 or p39 increased both the number of functional synapses and the frequency of spontaneous neurotransmitter release.

Insulin release from the pancreatic beta cell is important for regulating blood glucose levels and in Type II diabetes this process is impaired. We have previously identified Cdk5 as an enhancer of beta cell exocytosis and now show that both Cdk5 activators are expressed in mouse primary beta cells and associate with membranes. Munc18-1 is a Cdk5 substrate essential for neuronal exocytosis participating in SNARE complex assembly. Capacitance recordings using phosphorylation mutants showed that Munc18-1 is a substrate of Cdk5/p39, promoting Ca^{2+}-dependent beta cell exocytosis. Munc18-2 is preferentially expressed in cells not expressing Munc18-1. We demonstrated that Munc18-2 is expressed in pancreatic beta cells, in addition to Munc18-1. Munc18-2 showed a more cytosolic localization and Munc18-1 was selectively translocated to the plasma membrane after glucose-stimulation. Phosphorylation mutants of Munc18-1 and Munc18-2 showed different subcellular localization compared to wt proteins, implicating that phosphorylation is important for Munc18 cycling.

Thus, we have shown that *in vivo* the switch from SNAP-25a to SNAP-25b is essential for developing and maintaining accurate synaptic performance in plastic brain areas. Cdk5 promotes exocytosis in both neuronal and pancreatic beta cells, in the latter via the p39 activator. Furthermore, we have identified Munc18 proteins as Cdk5 substrates in beta cells and demonstrated a late role for Cdk5 phosphorylation of Munc18-1 in exocytosis.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which in the text will be referred to by their Roman numerals:

I. **Johansson JU**, Ericsson J, Janson J, Beraki S, Stanic D, Mandic SA, Wikström MA, Hökfelt T, Ögren S-O, Rozell B, Berggren P-O, Bark C. Genetic elimination of SNAP-25b results in altered synaptic plasticity, seizure activity and cognitive impairment. *Submitted*

II. **Johansson JU**, Lilja L, Chen X-L, Higashida H, Meister B, Noda M, Zhong Z-G, Yokoyama S, Berggren P-O, Bark C. (2005) Cyclin-dependent kinase 5 activators p35 and p39 facilitate formation of functional synapses. *Brain Res Mol Brain Res.* 138:215-27

III. Lilja L, **Johansson JU**, Gromada J, Mandic SA, Fried G, Berggren P-O, Bark C. (2004) Cyclin-dependent kinase 5 associated with p39 promotes Munc18-1 phosphorylation and Ca(2+)-dependent exocytosis. *J Biol Chem.* 279:29534-41

CONTENTS

LIST OF ABBREVIATIONS

Ach	Acetylcholine
aCSF	Artificial cerebrospinal fluid
ADHD	Attention deficit hyperactivity disorder
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BDNF	Brain-derived neurotrophic factor
BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CCK	Cholecystokinin
Cdk5	Cyclin-dependent kinase 5
CNS	Central nervous system
CRMP-2	Collapsin response mediator protein-2
dBcAMP	Dibutyryladenosine cyclic monophosphate
DAP-kinase	Death Associated Protein kinase
DCx	Doublecortin
DMEM	Dulbecco´s modified Eagle medium
Dn	Dominant negative
DOC2	Double C2-domain
ECL	Enhanced chemiluminescence
eGFP	Enhanced green fluorescent protein
EPSC	Evoked excitatory postsynaptic current
ERK	Extracellular-signal-regulated kinase
GABA	γ-aminobutyric acid
GAP43	Growth-associated protein-43
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GEF	GDP/GTP exchange factor
GFP	Green fluorescent protein
GLUT	Glucose transporter
GTP	Guanosine triphosphate
hGH	Human growth hormone
HRP	Horse-radish peroxidase
IPI	Interpulse interval
KO	Knock-out
KRBH	Krebs-Ringer bicarbonate HEPES buffer
LDCV	Large dense core vesicle
MAP	Microtubule-associated protein
mEPP	Miniature endplate potential
Mint	Munc18-interacting protein
Munc13	Mammalian homolog of the *unc*-13 gene
Munc18	Mammalian homolog of the *unc*-18 gene
NGF	Nerve-growth factor
NMDA	N-methyl d-aspartate
NMJ	Neuromuscular junction

NPY	Neuropeptide Y
NSF	N-ethylmaleimide-sensitive factor
PBS	Phosphate-buffered saline
PIP_2	Phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase
PKC	Protein kinase C
PLD	Phospholipase D
PN	Postnatal
PSD-95	Post-synaptic density protein-95
PPF	Paired-pulse facilitation
PVDF	Polyvinylidene difluoride
RIM	Rab3-interacting molecule
RRP	Ready releasable pool
RT	Room temperature
RT-PCR	Reverse transcriptase-polymerase chain reaction
SM	Sec1/Munc18
SNAP	Soluble NSF attachment protein
SNAP-25	Synaptosomal-associated protein of 25 kD
SNARE	Soluble NSF attachment protein receptor
SV	Synaptic vesicle
TAE	Tris-acetate-EDTA
TBE	Tris-borate EDTA
VAMP	Vesicular-associated membrane protein
VDCC	Voltage-dependent Ca^{2+}-channel
Wt	Wildtype

1 INTRODUCTION

1.1 EXOCYTOSIS

1.1.1 The exocytotic process

The way by which cells release substances through fusion of vesicles with the plasma membrane is called exocytosis. Regulated exocytosis is a special feature of secretory cells and comprises the release of neurotransmitters, peptides and hormones. There is a series of discrete biochemical steps leading to release of signal substances and the final stages in exocytosis are classified into docking, priming and fusion, all of which are ATP- and Ca^{2+}-dependent (Becherer & Rettig, 2006; Rorsman & Renström, 2003; Südhof, 2004). Vesicles are recruited and tethered to the plasma membrane where they are docked. During priming *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptor (SNARE) complexes are formed, vesicles are pulled closer to the plasma membrane and mature into fusion-competence. At the fusion step a fusion pore is formed by the mixing of vesicle and plasma membrane lipid bilayers and vesicle content is released into the extracellular space (Margittai et al., 2003). Although SNARE pairing and SNARE-SNARE interactions were initially proposed to provide the overall specificity and mediate membrane trafficking and fusion, it is now clear that there are additional proteins needed to provide accuracy and speed of exocytosis. The exocytotic process is intensely orchestrated, with many players involved. Some proteins may be necessary to keep exocytosis fine-tuned and serve as brakes, though at the end being necessary for exocytosis to function properly.

1.1.2 The SNARE complex

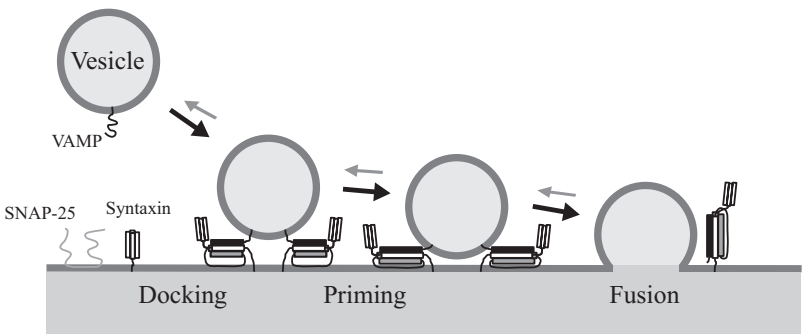

Figure 1. The steps of exocytosis and the formation of the SNARE complex. Vesicles are translocated to the plasma membrane where they are docked. Priming of vesicles involves formation of SNARE complexes. Prior to assembly of the SNARE complex SNAP-25 and VAMP has an unordered structure, thereafter adopting the characteristic SNARE conformation. Finally, release occurs when vesicles fuse with the plasma membrane.

The molecular machinery acting in exocytosis is similar for all cellular vesicle trafficking steps. Central for exocytosis is the formation of a trans-membrane soluble SNARE complex (Söllner et al., 1993a; Söllner et al., 1993b). The SNARE complex consists of three compartmentally defined proteins; the Vesicular-associated membrane protein (VAMP)/Synaptobrevin, the target membrane anchored Syntaxin and the

cytoplasmic Synaptosomal-associated protein of 25 kD (SNAP-25) that associates with membranes through palmitoylation (Archer et al., 1990; Bennett et al., 1992). The so called SNARE motif each consists of 60-70 amino acids and four SNARE motifs are forming a four α-helix coiled-coiled bundle, pulling the vesicle close to the target membrane (Sutton et al., 1998). VAMP and Syntaxin have one SNARE motif each adjacent to their C-terminal trans-membrane region and SNAP-25 has one N-terminal and one C-terminal SNARE motif connected by a palmitoylated linker. The assembly of the SNARE complex is believed to initiate at the N-terminal of the motifs, proceeding towards the C-terminus. The interface between the helices is mainly hydrophobic except for one charged amino acid of each SNARE motif, arginine (R) or glutamine (Q). The SNAREs are classified into R (VAMP) and Q (Syntaxin and SNAP-25) SNAREs according to which of these residues they contain (Fasshauer et al., 1998). The mechanisms mediating formation of the initial fusion pore are still not fully elucidated (Jahn & Scheller, 2006; Südhof, 2004). However, the SNARE proteins are believed to operate at the actual fusion event and have intrinsic, albeit slow, capabilities to perform membrane fusion (Giraudo et al., 2006; Pobbati et al., 2006). Cleavage of the SNARE proteins by clostridial neurotoxins abolishes release and have demonstrated the necessity of the SNARE complex in fusion (Schiavo et al., 2000). After assembly of the SNARE complex Complexin rapidly binds along the α-helix bundle (Chen et al., 2002; Tang et al., 2006). The ATPase NSF and soluble NSF attachment proteins (SNAPs) dismantle the SNARE complexes after fusion (Brunger, 2005). There exist several closely related protein variants for the SNARE proteins, either obtained through alternative splicing of different exons from the same gene or by expression from similar but distinct genes (Hong, 2005). In general, higher evolved organisms exhibit a higher number of protein isoforms. These protein variants, also referred to as isoforms, provide SNAREs for various intracellular trafficking steps as well as varying modes of exocytosis at the plasma membrane. Syntaxins exhibits the greatest number of isoforms with Syntaxin 1-4 showing plasma membrane preference. Some Syntaxins, including Syntaxin 1, also has an N-terminal three-helix bundle called the Habc domain, that when folded onto the SNARE motif hinders formation of SNARE complexes (Brunger, 2005). Expression of synaptic proteins and their alternatively spliced isoform variants is strictly regulated, both anatomically and temporally (Elferink et al., 1989; Hepp & Langley, 2001; Shimohama et al., 1998).

1.1.3 SNAP-25

The classical SNARE complex in regulated exocytosis consists of SNAP-25, Syntaxin 1 and VAMP2. SNAP-25 interacts with Syntaxin 1 at the plasma membrane and participates in docking and initiation of priming prior to association with VAMP2 (Nagy et al., 2005; Sørensen et al., 2003). SNAP-25 null mutant mice exhibit embryonic lethality and evoked synaptic transmission is abolished. Despite the complete absence of SNAP-25, early nervous system development and spontaneous transmitter release are not affected (Molnar et al., 2002; Washbourne et al., 2002). SNAP-25 has two splice variants, SNAP-25a and SNAP-25b, which differ in the central palmitoylated domain that is responsible for membrane targeting (Bark, 1993; Bark & Wilson, 1994; Gonzalo et al., 1999; Lane & Liu, 1997). SNAP-25a expression precedes the expression of SNAP-25b during neuronal development but in adult brain SNAP-25b comprises more than 90% of total SNAP-25 levels (Bark et al., 1995;

Boschert et al., 1996). However, SNAP-25a expression remains in certain brain regions, such as some cortical and hypothalamic structures, and in endocrine and most neuroendocrine cells SNAP-25a is the dominant isoform (Bark et al., 1995; Boschert et al., 1996; Grant et al., 1999). A number of SNAP-25 homologs have been identified that are involved in slower modes of exocytosis; SNAP-23/Syndet, SNAP-29 and SNAP-47 (Chieregatti et al., 2004; Holt et al., 2006; Pan et al., 2005).

1.1.4 Sec1/Munc18 (SM) proteins

The Sec1/Munc18 (SM) protein family is involved in controlling all intracellular vesicle trafficking steps. Three mammalian isoforms of Mammalian homolog of the *unc*-18 gene (Munc18) have been identified; Munc18-1 (with two alternatively spliced variants, Munc18-1a and Munc18-1b), Munc18-2 and Munc18-3 (Garcia et al., 1994; Hata et al., 1993; Hata & Südhof, 1995; Pevsner et al., 1994; Tellam et al., 1995). Munc18-1 appears to have a unique role in neurotransmitter release as null mutant mice show completely abolished neurotransmitter release (Verhage et al., 2000). It has been hard to reconcile the precise function(s) of Munc18-1 but it is becoming clear that this protein is involved in several steps of the exocytotic pathway. This may explain the conflicting data generated regarding whether Munc18-1 is auxiliary or inhibitory (Dresbach et al., 1998; Schulze et al., 1994; Zhang et al., 2000). One of the proposed functions of Munc18-1 is to hold Syntaxin 1 in a closed conformation, thereby preventing Syntaxin 1 to participate in SNARE complex formation (Misura et al., 2000). At the plasma membrane Syntaxin 1 fluctuates between a closed, to which Munc18-1 binds with high affinity, and an open conformation prior to SNARE complex formation (Margittai et al., 2003). Munc18-1 is the only SM protein binding to the closed conformation of Syntaxin 1 (Toonen & Verhage, 2003). In neuroendocrine cells but not in neuronal cells, Munc18-1 is essential for docking of vesicles. A more general feature of SM proteins is to bind to the N-terminal domain of Syntaxins, suggesting a more transient interaction and a function in SNARE complex assembly. It has been suggested that SM proteins confer specificity to SNARE complexes and Munc18-1 specifically activates Syntaxin 1/SNAP-25/VAMP2/3 formation (Shen et al., 2007; Toonen & Verhage, 2003). It was only recently shown that Munc18-1 is stimulating fusion when binding to the assembling SNARE complex (Dulubova et al., 2007; Rickman et al., 2007; Shen et al., 2007; Zilly et al., 2006). Munc18-1 might stabilize a half-open conformation of Syntaxin 1 and attract VAMP2. Importantly, SNARE pairing must be initiated before this action of Munc18, thus separating it from the docking function of Munc18-1. Munc18-2 expression is prominent in non-neuronal epithelial and mast cells where it controls secretion (Martin-Verdeaux et al., 2003; Riento et al., 2000). Munc18-3 is a partner of Syntaxin 4 in controlling insulin stimulated translocation of the Glucose transporter-4 (GLUT-4) in muscle and adipose tissue and in amylase release from parotid acinar cells (Imai et al., 2004; Ishiki & Klip, 2005; James, 2005).

1.2 RELEASE

1.2.1 Composition of the release site

Large scaffolding multidomain proteins such as Piccolo and Bassoon are involved in structuring the release site and organizing the trafficking of vesicles (Schoch & Gundelfinger, 2006). Rab3-interacting molecule (RIM) proteins interact with a number

of presynaptic proteins such as Rab3 and Mammalian homolog of the *unc*-13 gene (Munc13) proteins (Betz et al., 2001; Wang et al., 1997), and have an established role in priming (Kaeser & Südhof, 2005). The essential priming factor Munc13 is believed to mediate a conformational switch of Syntaxin necessary for SNARE complex formation (Brose et al., 2000). Rab proteins, in particular Rab3a in brain, are associated with vesicles in their GTP-bound state, mediate contacts with active zone proteins and dissociate from membranes during exocytosis (Schluter et al., 2002). A common trait of these proteins is that they appear to perform several actions in transmission. The actin cytoskeleton is prominent at the plasma membrane. Besides providing structure, actin is believed to be intimately linked to vesicle organization and trafficking and also serving as a scaffold holding proteins involved in several transduction pathways (Dillon & Goda, 2005; Goda & Davis, 2003).

1.2.2 Release stimulus

Ca^{2+} is an important second messenger and a local increase in Ca^{2+}-concentration is considered as the trigger for regulated exocytosis. Arrival of an action potential opens voltage-dependent Ca^{2+}-channels (VDCCs) and influx of Ca^{2+} causes release-competent vesicles to fuse with the plasma membrane. Neurotransmitter release involves primarily P/Q- and N-type Ca^{2+}-channels (members of the Ca_v2 channel family) (Evans & Zamponi, 2006), whereas hormone release relies on L-type Ca^{2+}-channels (Ca_v1 channels) (Yang & Berggren, 2005; , 2006). The Ca^{2+}-concentration at the site of release rises to the μM range, higher for neurotransmission than for hormone release, and accordingly Ca^{2+}-channels are tighter coupled to the release machinery in neurons (Zucker, 1996). Synaptotagmin I is believed to be the main Ca^{2+}-sensor for fast synchronous release in neurons, by binding to formed SNARE complexes and initiate fusion after Ca^{2+}-entry (Brose et al., 1992; Rizo et al., 2006). The N-terminal transmembrane domain of Synaptotagmin I is typically attached to vesicles and via two Ca^{2+}-binding C2 domains it interacts with vesicle and plasma membranes (Bai & Chapman, 2004). There are several isoforms of Synaptotagmins with different Ca^{2+}-affinities and some of them are plasma membrane located, providing cells with a bouquet of Ca^{2+}-sensors (Südhof, 2002). The Ca^{2+}-sensors in pancreatic beta cells are more enigmatic, however a functional role for, in particular, Synaptotagmin IX has been demonstrated (Grise et al., 2007; Iezzi et al., 2004). Contrary to action potential-stimulated release, spontaneous release occurs in a potentially less regulated and Ca^{2+}-dependent manner and is not dependent on Synaptotagmin I (Geppert et al., 1994; Melia, 2007). Recently Synaptotagmin-12, which does not bind Ca^{2+} but is regulated by phosphorylation, was suggested to promote spontaneous release in mature neurons (Maximov et al., 2007). Interestingly, spontaneous release seems to increase when evoked release is disrupted and may indicate the recruitment of vesicles with other components. Similar to spontaneous release of neurotransmitters, peptides and hormones, constitutive exocytosis involves the continuous addition of lipids and proteins into the plasma membrane. Many other synaptic proteins contain C2 domains, which generally confers affinity for phospholipids in a Ca^{2+}-dependent or -independent manner (Rizo & Südhof, 1998). Phospholipids, and in particular phosphatidylinositol 4,5-bisphosphate (PIP_2), are important for exocytosis, both as precursors of signaling molecules, including diacylglycerol, and through interactions with proteins (Di Paolo & De Camilli, 2006). Phospholipids provide links to two crucial requirements in

exocytosis, Ca^{2+}-signaling and membrane proximity and regulation. For instance Rabphilin3, RIM, Double C2-domain (DOC2) and Munc13 mediate Ca^{2+}-dependent actions in exocytosis, in the case of Munc13 via Calmodulin (Groffen et al., 2006; Junge et al., 2004; Oishi et al., 1996; Wang & Südhof, 2003).

1.2.3 Neurotransmission

Classical neurotransmitters are predominantly released by synaptic vesicles (SVs) at discrete sites called active zones in chemical synapses. Neurotransmitters are considered to be released in quanta, i.e. fusion of one vesicle denotes the release of one quanta as defined by electrophysiologically measured postsynaptic currents (Del Castillo & Katz, 1954). Strength and efficacy of synaptic transmission change continuously as a result of activity-dependent changes throughout development and during neuronal rewiring in the mature nervous system, a process denoted as plasticity. Short-term plasticity reflects the properties of a synapse to adapt to changes in response to Ca^{2+} without long-lasting effects (Zucker & Regehr, 2002). Long-term plasticity is required for memory and learning and is induced by postsynaptic Ca^{2+}-influx (Lamprecht & LeDoux, 2004). It requires gene expression and structural reorganization and via retrograde signaling results in activation and recruitment of new synapses and increased release. The strength of a synaptic connection is reflected by the number of release sites, the probability of transmitter release at each site and the amplitude of the postsynaptic response (Choi et al., 2000). Expression of different protein isoforms can provide cells with differential plastic properties (Basu et al., 2007; Kaeser & Südhof, 2005; Rosenmund et al., 2002).

1.2.3.1 The neuromuscular junction

The neuromuscular junction (NMJ) where motor neurons synapse on muscle cells has been extensively studied as it is more easily accessible than central synapses (Goda & Davis, 2003). The main neurotransmitter at the NMJ is acetylcholine (ACh) that binds to postsynaptic nicotinic or muscarinic ACh receptors. In essence, transmission at the NMJ resembles that of central synapses but differs in some aspects. For instance, NMJ synapses depend on co-expression of Synaptotagamin I and II for evoked transmission and during development transmission appears more important than for central synapses (Goda & Davis, 2003; Pang et al., 2006).

1.2.4 The hippocampus

The hippocampus in the mesolimbic area of the brain is involved in cognitive function and in particular learning and memory formation. Accordingly, it is a highly plastic area able to change with altered neuronal activity. The dentate subgranular zone of the hippocampus is one of few areas in brain where neurogenesis continues throughout adulthood (Kee et al., 2007; van Praag et al., 2002). In the hippocampus, excitatory synapses generally reflect synapses releasing the neurotransmitter glutamate and inhibitory synapses reflect GABA (γ-aminobutyric acid) release (Kandel et al., 2000). At the postsynaptic site they generate excitatory currents via AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid) and NMDA (N-methyl d-aspartate) receptors and inhibitory currents via GABA receptors. The major excitatory pathway in the hippocampus originates in the hilar region of the dentate gyrus with the granule

cells, receiving input from the entorhinal cortex. Granule cells project large vesicle-filled mossy fiber terminals with multiple discrete active zones onto CA3 pyramidal neurons and contact inhibitory interneurons by axonal *en passant* boutons (Galimberti et al., 2006). CA3 neurons then form Schaffer collateral synapses onto CA1 pyramidal neurons (Charlton et al., 1982; Katz & Miledi, 1968).

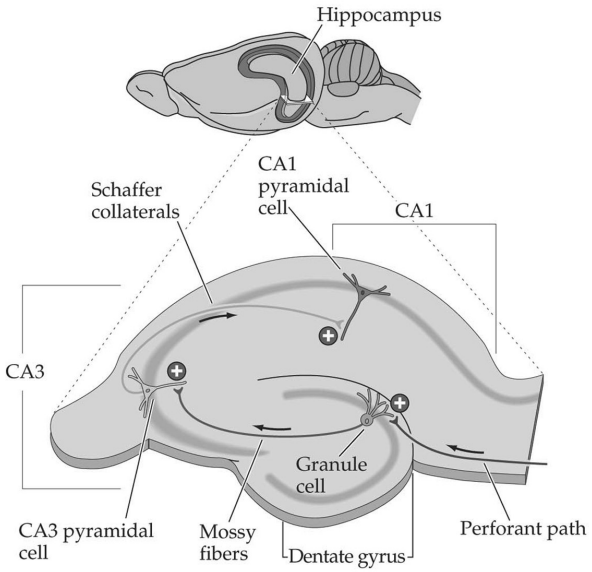

Figure 2. The major excitatory pathway in the hippocampus and its location in rodent brain. Figure is from (Purves et al., 2004) with permission from the publisher.

1.2.5 Insulin release

Hormones are stored in large dense core vesicles (LDCVs) or secretory granules in neuroendocrine and endocrine cells and released upon stimulation. Similarly, in neurons neuropeptides and neurotrophins are released from LDCVs (Hökfelt, 1991). Classical neurotransmitters are also stored in LDCVs/granules together with these substances, allowing co-transmission. Although the exocytotic machinery is similar for neurotransmitter release and for hormone secretion there are also differences (Neher, 2006; Rorsman & Renström, 2003). There are no discrete release sites for hormone release but fusion occurs in the vicinity of Ca^{2+}-channels. Hormone release requires a higher magnitude and frequency of stimulation before fusion than neurotransmitter release. The slower exocytosis mode has proven to be very useful in studying exocytotic mechanisms that have been difficult to reconcile in fast neurotransmission. Furthermore, it allows direct measurements of release from the secreting cell, instead of indirect postsynaptic measurements, due to the large size of the LDCVs/granules and thereby increases in membrane area after secretion. In the pancreas, beta cells from the islets of Langerhans release insulin from secretory granules to maintain glucose homeostasis in the body, through a process denoted as stimulus-secretion coupling (Ashcroft et al., 1994; Berggren & Larsson, 1994; Lang, 1999). Elevation of blood

glucose levels induces glucose uptake into beta cells through GLUTs. Glucose metabolism thereafter increases the ATP/ADP ratio in the cell and this allows for closure of K_{ATP}-channels, resulting in depolarization of beta cells and opening of L-type VDCCs and Ca^{2+}-entry. Insulin release exhibits a biphasic pattern and only the first phase of insulin release can be induced by K^+, whereas the second phase requires stimulation by secretagogues such as glucose and metabolism (Gembal et al., 1992; Rorsman & Renström, 2003; Straub & Sharp, 2002). In addition, an amplifying pathway involving cAMP but not directly the K_{ATP}-channel appears to operate in beta cells to mediate insulin release (Gembal et al., 1992). In Type II diabetes mellitus in particular the first phase of insulin release is impaired and a reduction in expression of exocytotic proteins has been noted (Del Prato et al., 2002; Zhang et al., 2002).

1.2.6 Vesicle pools and trafficking

Regulated exocytosis exhibits a remarkable variation in Ca^{2+}-sensitivity, rates of fusion, latencies to fusion and persistence of secretion (Martin, 2003; Zucker, 1996). Release occurs in a biphasic mode. The first phase consists of a fast and a slow component, followed by lower, sustained release. All these steps are faster for exocytosis of SVs than for LDCVs/granules (Martin, 2003). For instance, the fast component of the first phase, called the exocytotic burst, lasts 1 ms for SVs and 1000 ms for beta cell granules. Vesicles are categorized to belong to different pools depending on their availability to be released (Becherer & Rettig, 2006). Normally, only few vesicles are fusion-competent and require no further modifications prior to exocytosis. These vesicles are referred to as the readily releasable pool (RRP) and are released during the burst phase. Remaining vesicles need to undergo both ATP- and Ca^{2+}-dependent maturation steps in order to be mobilized to the RRP (Becherer & Rettig, 2006; Rorsman & Renström, 2003; Südhof, 2004). This process, called priming, is required for sustained release and is believed to recruit preferentially already docked vesicles. It has been shown that the primed pool of LDCVs is unstable in contrast to SVs and requires ATP (Heidelberger et al., 2002; Xu et al., 1998). Besides utilizing other protein isoforms than for SVs, certain proteins appear to be more important for LDCV/granule exocytosis, such as Ca^{2+}-dependent activator protein for secretion (CAPS) (Wassenberg & Martin, 2002). By binding PIP_2 in the plasma membrane and interacting with the vesicle membrane, CAPS participates in priming (Grishanin et al., 2004; Olsen et al., 2003). In central hippocampal synapses the size of the RRP of SVs has been estimated to ~5-10 and that correlates with the number of fully docked vesicles (Dobrunz & Stevens, 1997; Schikorski & Stevens, 2001). In contrast, the docked pool of LDCVs/granules is large, concomitant with a lesser requirement for fast recycling and pool replenishment. Whereas SVs are refilled in the synapse during local recycling, hormones are taken up into granules via the Golgi pathway (Lang, 1999; Südhof, 2004). In pancreatic beta cells ~2000 granules are located close to the plasma membrane; ~600 are docked of which ~50 belong to the RRP (Barg et al., 2002a). Vesicles located further away from the plasma membrane are considered belonging to reserve pools. It is not fully established whether vesicles must undergo the sequential steps via docking prior to fusion or whether they can be recruited directly (Neher, 2006; Rizzoli & Betz, 2004). For example, the second phase of insulin release has been suggested to be mediated by non-docked, newly recruited granules (Ohara-Imaizumi &

Nagamatsu, 2006). Furthermore, the sequential steps of exocytosis are not irreversal (Nagy et al., 2004).

Actin seems to serve a dual role, both providing tracks for vesicles and acting as a barrier (Dillon & Goda, 2005; Malacombe et al., 2006). In central neurons there is less evidence for actin in vesicle trafficking, though actin cytoskeleton appears to reorganize in response to activity (Dillon & Goda, 2005). The lack of discrete release sites and the large number of vesicles located in the vicinity of the plasma membrane suggest a more active role of actin in non-neuronal cells. There are differences in the docking process compared to neuronal cells and it may involve the actin motor protein Myosin V (de Wit et al., 2006; Rutter & Hill, 2006; Toonen et al., 2006a). Stimulation of beta cells also induces granule movement more distal from the plasma membrane, dependent on the microtubule cytoskeleton and the motor protein Kinesin-1 (Rutter & Hill, 2006).

1.2.7 Fusion modes and vesicle retrieval

Exocytosis needs to be balanced by membrane retrieval in a process called endocytosis. Fusion can occur in mainly two different modes (Harata et al., 2006). At full fusion the vesicles merge completely with the plasma membrane. Endocytosis after this event is slow and involves Clathrin coating of vesicles and recycling through endosomes. Alternatively, vesicle content can be released through the fusion pore and vesicles pinched off without full mixing of membranes, which is referred to as kiss-and-run exocytosis. It is becoming clear that the mode of endocytosis can contribute to synaptic strength but the extent of the two modes is not established (Harata et al., 2006; Rutter & Hill, 2006). In particular kiss-and-run exocytosis may be functioning in neurotransmitter release, as SVs are small and opening of a fusion pore would empty the whole vesicle of small neurotransmitters. Retrieval of vesicles without full fusion can speed up recycling which is important for synapses with small vesicle pools (Südhof, 2004). Modifying the conductance of the fusion pore can be a way of regulating quantal content (Barclay et al., 2004). In beta cells insulin release through the fusion pore seems negligible and most likely requires full fusion, though kiss-and-run exocytosis has been suggested to have a functional role (Barg et al., 2002b; Rutter & Hill, 2006).

1.3 PROTEIN PHOSPHORYLATION AND DEPHOSPHORYLATION

Phosphorylation by protein kinases provides a fast and powerful modification of proteins. The addition of a phosphate group to serine, threonine or tyrosine residues changes the properties of a protein, for example by activating or inactivating it, by inducing a change in conformation or affinity for other proteins. Phosphorylation affects for instance vesicle pool sizes and phosphorylation of ion channels may modulate channel conductance and neuronal activity (Leenders & Sheng, 2005; Snyder et al., 2006). The second messenger-activated serine/threonine kinases cAMP-dependent protein kinase (PKA) and Protein kinase C (PKC) are prominent in secretion (Leenders & Sheng, 2005). PKA phosphorylates for instance α-SNAP, Synapsin I, Snapin and RIM1 (Lonart et al., 2003; Snyder et al., 2006). The conventional PKC isoforms α, β and γ are activated by diacylglycerol and Ca^{2+} and Munc18-1, Synaptotagmin I, NSF and Ca^{2+}-channels are PKC substrates (Morgan et al., 2005; Snyder et al., 2006). Ca^{2+}/Calmodulin-dependent protein kinase II (CaMKII) is mostly

connected with postsynaptic plasticity but presynaptically CAMKII phosphorylates VAMP2, Synaptotagmin I, Synaptophysin and N-type Ca^{2+}-channels (Leenders & Sheng, 2005; Snyder et al., 2006). Other kinases phosphorylating synaptic proteins are Casein kinase I (CKI) (Syntaxin 1, Synaptotagmin I) and CKII (Syntaxin 1, VAMP2) (Snyder et al., 2006). Cyclin-dependent kinase 5 (Cdk5) is one of few members in the serine/threonine protein kinase family of Cdks that has only been implicated in mechanisms unrelated to cell cycle control (Meyerson et al., 1992; Smith et al., 2001). For activity, Cdk5 is dependent on association with one of its regulatory proteins, p35 or p39 (Lew et al., 1994; Tang et al., 1995; Tsai et al., 1994). Cdk5 is ubiquitously expressed in mammalian tissues, but expression of the activators is enriched in brain. Cdk5 is important for neuronal development but more recently a number of synaptic substrates have been identified (see the results and discussion section).

To balance phosphorylation, phosphate groups are removed by protein phosphatases (PPs) and this is equally important in regulation of protein function. The abundant serine/threonine phosphatases PP1, PP2A and PP2B belong to the large family of phosphoprotein phosphatases and participate in secretion (Sim et al., 2003). A large number of subunits of these phosphatases provide diversity in substrate recognition and much remains to be learnt about the substrates of phosphatases. The activity of phosphatases is regulated by other proteins, including kinases. Calcineurin/PP2B is the only phosphatase sensitive to Ca^{2+}/Calmodulin and this phosphatase is particularly active in endocytosis.

2 AIMS

This thesis aimed at describing release regulatory mechanisms in different mammalian systems, from single cells to mice. Focus was on two aspects of exocytotic regulation; protein isoforms and phosphorylation. Specifically, the following was investigated:

- The *in vivo* importance of the SNAP-25b isoform for brain function through characterization of a gene targeted mouse mutant exclusively expressing SNAP-25a.

- The roles of Cdk5 phosphorylation and activator proteins p35 and p39 in exocytosis in spontaneous neurotransmitter release, in an *in vitro* model of the neuromuscular junction, and in insulin secretion from pancreatic beta cells.

- Munc18 proteins as Cdk5 substrates and as regulators of exocytosis in beta cells.

3 METHODOLOGIES

The basis for all studies in this thesis has been molecular biology techniques performed by me and first authors of the included publications and preliminary results. Collaborations have been carried out to improve the functional understanding of exocytosis.

3.1 ANIMALS AND CELLS; ISOLATION AND CULTURE

All animal breeding and animal studies were done in accordance with the guidelines from the local ethical committees. Cell culturing was carried out at 37°C in a humidified atmosphere with 5 % CO_2.

3.1.1 SNAP-25b null mice

The SNAP-25b null mice were generated by targeted gene replacement in embryonic stem (ES) cells followed by positive selection for neomycin resistance (Mansour et al., 1988). ES clones exhibiting a true homologous recombination were injected into blastocysts that subsequently were implanted into pseudopregnant C57BL/6NCrl females. Chimeric offspring demonstrating germline transmission were used as founders, backcrossed on C57BL/6NCrl background until congenic and the neomycin gene was excised using Prm1Cre mice that express the Cre recombinase in testis (O'Gorman et al., 1997). Thereafter the Cre transgene was crossed out from the floxed SNAP-25b null mouse mutant lines.

3.1.2 Brain

Mouse brain was isolated after terminal CO_2 anesthesia and tissue frozen in liquid N_2 for RNA and protein analyses. In paper III, RNA was isolated from C57BL/6NCrl mouse brain and *ob/ob* mouse brain was the source of RNA used in preliminary results.

3.1.3 Muscle cells and co-cultures

Newborn Wistar rats were decapitated and then rinsed in 70% ethanol. Rat hindlimb muscle cells, isolated by trypsinization, were cultured in Dulbecco´s modified Eagle medium (DMEM) for 7 days and grown to form myotubes 20-30 μm in diameter and >100 μm long. Transfected NG108-15 cells were overlaid on the already fused and contracting muscle cells. The co-cultures were maintained in DMEM supplemented with 10% horse serum and 0.25 mM dibutyryladenosine cyclic monophosphate (dBcAMP).

3.1.4 Primary beta cells

Primary beta cells from 10-12 months old *ob/ob* mice were used for detection of endogenous expression. *Ob/ob* islets are relatively large and composed of more than 90% beta cells (Hellman, 1965; Nilsson et al., 1987). For electrophysiological recordings female NMRI mice were used. Pancreatic islets were isolated by collagenase digestion as previously described (Lernmark, 1974; Nilsson et al., 1987). Single cells were prepared by shaking in a Ca^{2+}-free solution. Cells were cultured for up to 3 days in RPMI 1640 culture medium containing 11 mM glucose supplemented

with 10% (v/v) heat-inactivated fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine. For subcellular fractionation primary beta cells were incubated for 1 h in supplemented RPMI 1640 medium containing 3 or 17 mM glucose.

3.1.5 Cell lines

The NG108-15 cell line, a hybrid between the mouse neuroblastoma cell line N18TG2 and the rat glioma cell line C6-BU-1, was cultured in DMEM, supplemented with 10% fetal calf serum, 100 IU/ml penicillin, 100 μg/ml streptomycin and 4 mM L-glutamine. Differentiation was induced by culturing the cells in 0.5 mM dBcAMP (Sigma) for 5-7 days. The NG108IIA1 cell line (NG108-15 cells stably overexpressing Synapsin IIa) was cultured using the same conditions.

The mouse insulinoma MIN6 cell line was cultured in DMEM supplemented as for culture of primary beta cells including 64 μM β-mercaptoethanol. For subcellular fractionation cells were starved for 1h in Ca-5 buffer (1.25 M NaCl, 59 mM KCl, 1.28 mM $CaCl_2$ and 12.0 mM $MgCl_2$) and incubated under either unstimulatory (0.5 mM glucose, DMEM) or stimulatory (25 mM glucose, DMEM) conditions for 30 min.

The rat insulinoma INS-1E cell line was cultured in RPMI 1640 medium supplemented with 5% fetal calf serum, 1 mM sodium pyruvate, 50 μm 2-mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 100 U/ml penicillin and 100 μg/ml streptomycin.

3.1.6 Transfections

Primary beta cells, NG108-15 cells in mono-culture, MIN6 cells and INS-1E cells were transfected using the Lipofectamine 2000 technique (Invitrogen). After transfection cells were cultured for 48-72 h, except for antisense oligonucleotide treated cells (24 h). For co-cultures NG108-15 cells were transfected using Lipofectamine or Lipofectamine plus (Invitrogen), harvested 1 day after transfection and co-cultured with myotubes for 1-7 days.

Expression vectors for preliminary results. Flag- and Myc-tagged Munc18-1 constructs were obtained by PCR amplification of cDNA encoding the rat Munc18-1 wt sequence with three different and overlapping forward primers comprising tag sequences (in bold) and restriction sites for BamHI and EcoRI (underlined). For Flag, following forward primers were used: primer 1, 5' – tgaattcgccaccatg**gattacaaggatgac** – 3'; primer 2, 5' – g**gattacaaggatgacgacgataag**ggaggtgc – 3' and primer 3, 5' – **gataag**ggaggtgcccccattggcctcaag – 3'. Myc-tag primers were: primer 1, 5' – tgaattcg ccgccatg**gagcagaaactcatctc** – 3'; primer 2, 5' – **gagcagaaactcatctctgaagaggatctg**g gag – 3' and primer 3, 5' – **gaagaggatctg**ggaggtgcccccattggcctcaag – 3'. Reverse primer for all PCR reactions was 5' - cgcttgttgatatcctccacaattgtg - 3'. To achieve a more efficient translation initiation the first forward primers were modified to generate a Kozak sequence. Generated PCR products were cloned into pIRES2-EGFP vector using EcoRI/BamHI. For generation of a Munc18-1 Cdk5 phosphorylation mutant, part of the Munc18-1 sequence containing the $Thr^{574} \rightarrow Ala^{574}$ mutation was cut out with EcoRV/ApaI from the pIRES2-EGFP construct from paper III, and inserted into Munc18-1 wt YFP-C1 (Clontech), originally cloned using HindIII/ApaI (a generous gift from Dr. Y. Liu, unpublished). Construct encoding rat Munc18-2 wt in EGFP-C3 (Clontech) was provided by Dr U. Blank (Martin-Verdeaux et al., 2003). Munc18-2

mutated in a potential Cdk5 phosphorylation site, Thr ^{573}→ Ala^{573}, was created by PCR amplification of DNA encoding rat Munc18-2 wt (pCMVmyc Munc18-2 wt construct, a kind gift from Dr. T. Südhof (Hata & Südhof, 1995)). Forward primer was, 5' – aatt*gaattc*caggcgccctt ggggctgaag – 3' (EcoRI site in italics) comprising the ATG start codon, and 5 µl of the PCR product from the following primers: forward, 5' – ataggctcttcccacatcctc**gca**ccaac – 3' (mutated site in bold) and reverse, 5' – gagggagaggggc*ggatcc* – 3' (BamHI site in italics) represents the reverse primer in the final PCR amplification reaction. The amplified product was subcloned into pGEM-Teasy vector (Promega) and thereafter inserted into a pEGFPC-1 vector (Clontech) (EcoRI/BamHI). The different constructs were DNA sequenced (ABI Prism 377) after amplification with the BigDye Terminator v3.1 Cycle Sequencing Kit (both Applied Biosystems).

3.2 RNA ANALYSES; RT-PCR

Total RNA was extracted using RNAeasy Mini Kit (Qiagen) or the GenElute^{TM} Mammalian Total RNA kit (Sigma). Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) was performed on 0.5-1 µg RNA using the SuperScript^{TM} RT-PCR System (Invitrogen) in a GeneAmp PCR System 9700 (Applied Biosystems). The amplified PCR products were separated by electrophoresis on 1-2% Tris-acetate-EDTA (TAE) agarose gels and visualized by ethidium bromide staining. 1-Kilobase DNA ladder (Invitrogen) was used as size marker. Purified gel fragments were sequenced as described above.

Preliminary results. RT-PCR was performed as described in the other papers with the exceptions that 39 cycles of amplification (20 cycles for semi-quantitative RT-PCR) and an annealing step of 56 ºC were used. For Munc18 the following primer pairs were used: Munc18-1a, 5' – tcgtccgcgtccttcagacac – 3' and 5' – tcagcgtgtccagcagtttc – 3'; for Munc18-1b, 5' – tcgtccgcgtccttcagacac – 3' and 5' – ctccattgttggagcctgatc – 3'; and for Munc18-2, 5' – ccagcatgccaacgtgcag – 3' and 5' – cttgaggtcatcgaggaagc – 3'. Amplification of Syntaxin isoforms were performed using Syntaxin specific primers (Kang et al., 2002) with one nucleotide modification in Syntaxin 2 antisense and Syntaxin 3 sense primers to match mouse Syntaxin cDNA sequences: Syntaxin 1A, 5' – atgaaggaccgaacccaggagc – 3' and 5' – tctatccaaagatgcccccga – 3'; for Syntaxin 2, 5' – atgcgggaccggctgccgg – 3' and 5' – tcatttgccaaccgacaagcc – 3'; and for Syntaxin 3, 5' – atgaaggaccggctggagcat – 3' and 5' – ttatttcagcccaacggacaatg – 3'.

3.2.1 Semi-quantitative RT-PCR

Since there are no antibodies available that can discriminate between the SNAP-25 isoforms, relative gene expression levels of SNAP-25a and SNAP-25b were determined from brain mRNA. RT-PCR was performed with 20 cycles of amplification and slightly adjusted annealing temperature for different primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primers were included in the reactions (25 µl) and a trace of [α^{32}P] dCTP (3000 µCi/mmol, PerkinElmer Life Sciences). The RT-PCR products were separated on 8% polyacrylamide Tris-borate EDTA (TBE) gels that were dried, and detected using a phosphoimager (BAS-1500, Fujifilm). Signal intensities were quantified using Image Gauge V3.45 (Fujifilm).

3.2.2 Determination of SNAP-25a/b mRNA ratio

Relative mRNA levels were solely determined at PN14. RT-PCR was run with SNAP-25 primers used for mRNA quantification in a different program. Amplified PCR products were EtOH precipitated and restriction enzyme digested with PvuI (restriction site exclusive for exon 5a) and StyI (site only present in exon 5b) for 2 h in separate reactions. The restricted RT-PCR products were separated on polyacrylamide gels and fragments quantified as described above.

3.3 PROTEIN ANALYSES; WESTERN BLOTTING

3.3.1 Whole-cell homogenates

Mouse brain and pancreatic islets were homogenized in buffer containing (in mM): 20 HEPES, 2 EDTA, 1 $MgCl_2$, and protease inhibitor cocktail (Roche Diagnostics GmbH), pH 7.4. SNAP-25 mouse brain homogenates were thereafter lyzed with 1% NP-40 (Sigma). NG108-15 cells were lyzed in 1% Triton X-100 PBS buffer with protease inhibitor cocktail. MIN6 cells were lyzed in 250 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 50 mM Tris-HCl pH 7.4 and protease inhibitor cocktail.

3.3.2 Soluble-membrane/cytoskeletal fractionations

Soluble/membrane fractionation of NG108-15 cells. Cells were homogenized in buffer containing (in mM): 20 HEPES, 2 EDTA, 1 $MgCl_2$ and protease inhibitor cocktail, pH 7.4. Samples were ultracentrifuged at 130,000 *x g* for 40 min and the supernatant was saved as the soluble fraction. Pellets were dissolved in 1% Triton X-100 and then centrifuged at 17,000 *x g* to remove cell debris.

Soluble/cytoskeletal fractionation of NG108-15 cells. Cells were lyzed in a 1% Triton X-100 buffer containing (in mM): 10 imidazole, 100 NaCl, 1 $MgCl_2$, 5 EDTA, 0.5 NaF, 0.1 μM okadaic acid and protease inhibitor cocktail, pH 7.4. To separate soluble and Triton X-100 insoluble (cytoskeletal-associated) fractions samples were ultracentrifuged at 130,000 *x g* for 20 min. The insoluble fraction was solubilized in RIPA buffer (5 mM NaCl, 1% NP-40, 0.1% sodium deoxycholate, 0.1% SDS, 50 mM Tris-HCl and protease inhibitor cocktail, pH 7.4).

Soluble/membrane fractionation of beta cell sucrose gradient fractions. Fraction 10 (with maximal amounts of p35) and fraction 12 (with maximal amounts of p39) from sucrose density gradients were diluted to a sucrose concentration of 0.25 M. Membrane-bound organelles were disrupted by freezing and thawing the diluted fractions five times. The membrane and soluble fraction of F10 and F12 were separated by ultracentrifugation at 100,000 *x g* for 1 h.

3.3.3 Sucrose density gradient fractionation

Brain (pulverized in liquid N_2), NG108-15 cells, islets or MIN6 cells were homogenized in (in mM): 20 HEPES, 1 $MgCl_2$, 250 D-sucrose, 2 EDTA and protease inhibitor cocktail, pH 7.4, and supplemented with glucose when appropriate. Homogenates were loaded onto a 4.4-ml linear sucrose density gradient (prepared from 0.6 and 2 M sucrose stock solutions). The gradient was centrifuged at 150,000 *x g* for 18 h and 15-16 fractions were collected from the top of the gradient. Percentage protein

in plasma membrane fractions was calculated using Syntaxin 1 or the Na^+/K^+ ATPase α-1 subunit as plasma membrane marker.

3.3.4 Gel electrophoresis and Western blotting

Protein concentrations were determined using the Bradford assay (Bio-Rad), with the exception that the bicinchoninic acid (BCA) assay from Pierce was used when lysis buffer contained SDS. Equal amounts of protein were separated on 10% Tris-glycine/NU-PAGE gels (Novex, Invitrogen) or 10% SDS polyacrylamide gels (preliminary results) and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Amersham Biosciences). Membranes were blocked in PBS containing 5% dry milk and 0.025% Tween 20 (VWR or Merck Eurolab) at room temperature (RT), probed with primary antibodies at 4°C and secondary antibodies at RT. For detection, membranes were treated with enhanced chemiluminescence (ECL plus, Amersham Biosciences) and exposed on film or scanned with a CCD camera (LAS 1000, Fujifilm). Quantification was done using the Image Gauge V3.45 software (Fujifilm).

Antibodies used for preliminary results. Antibodies directed to different tags were a mouse anti-c-Myc antibody (9E10, Santa Cruz Biotechnology) and a mouse monoclonal anti-Green Fluorescent Protein (GFP) antibody (JL-8, Clontech, BD Living Colors). A mouse anti-Munc18-1 antibody (Transduction Laboratories) and a rabbit anti-Munc18-2 antibody (a kind gift from Dr V. Olkkonen), directed against different Munc18 isoforms, were used. As a plasma membrane marker a mouse monoclonal anti-Na^+/K^+ ATPase α-1 antibody (C464, Upstate) was used.

3.4 HISTOLOGICAL AND IMMUNOCHEMICAL ANALYSIS

3.4.1 Histology and immunohistochemistry

Paper I: For histological analysis, PN14 and four month old mice were anesthetized with isoflurane and transcardially perfused with PBS followed by 4% paraformaldehyde in PBS. Organs were post-fixed overnight, dehydrated in ethanol and embedded in paraffin. 4 μm sections were stained with hematoxylin-eosin or cresyl violet. For immunohistochemistry in Figure 5 sections were collected on Superfrost Plus slides, deparaffinized in xylene, dehydrated in ethanol, and washed using Tris-buffered saline with Tween, pH 7.6. Sections were boiled by microwaving in 10 mM citrate buffer (pH 6.0, Merck) for antigen unmasking and blocked in 5% goat serum. Incubations of primary and secondary antibodies were done at 4°C and at RT, respectively. Slides were mounted in Vectashield (Vector Laboratories) or ProLong Antifade (Molecular Probes) and specimens observed as described for immunocytochemistry.

For neuropeptide immunohistochemistry PN14, PN21 and two months old mice were perfused with Ca^{2+}-free Tyrode's buffer, and a mixture of 4% paraformaldehyde and 0.2 % picric acid in 0.16 M phosphate buffer, pH 6.9. Brains were postfixed at 4ºC and immersed in 10% sucrose/0.01% sodium azide/0.02% Bacitracin/0.1 M phosphate buffer (pH 7.4) at 4ºC, before freezing with CO_2. 14 μm sections were thaw-mounted on aluminum gelatin-coated slides. Sections were incubated with primary antisera at 4ºC and to visualize CCK and NPY immunoreactivity, sections were processed using tyramide signal amplification (TSA^+, NEN Life Science Products). Secondary antibody

incubations were carried out at RT and sections were coverslipped using 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO, Sigma) in glycerol. Sections were examined and photographs taken using a Nikon Eclipse E600 fluorescence microscope (Nikon) and a Hamamatsu ORCA-ER C4742-80 digital camera, using Hamamatsu photonics Wasabi 150 software. For confocal analysis, a Bio-Rad Radiance Plus confocal scanning microscope installed on a Nikon Eclipse E600 fluorescence microscope was used with 488 nm argon and 543 nm HeNe excitation lasers. Images were processed using Adobe Photoshop software (Adobe Systems), as for immunocytochemistry below.

3.4.2 Immunocytochemistry

Paper II-III and preliminary results: Cells were fixed in 4% paraformaldehyde, permeabilized in 0.4% saponin and blocked in 10% goat serum/PBS (Sigma). Primary and secondary antibodies were applied at RT. For preliminary results a mouse monoclonal anti-Syntaxin antibody, clone HPC-1, and for detection of Flag-tagged Munc18-constructs a monoclonal anti-Flag, M2, antibody (both Sigma) were used. Coverslips were mounted in mounting media Vectashield or Mowiol. In paper II images were obtained using a Bio-Rad RadiancePlus laser scanning system (Bio-Rad) with excitation wavelengths 488, 543 and 638 nm. Images for paper III were obtained with a Leica TCS-SP2-AOBS confocal laser-scanner equipped with Argon and HeNe lasers connected to a Leica DMLFSA microscope (Leica Microsystems Heidelberg GmbH), using a 488, a 543 or a 633 laser in sequential scanning mode.

3.4.3 Determination of growth plate zones in hind limb

PN14 unfloxed SNAP-25b null hind limb specimen were fixed and stained with hematoxylin and eosin as described above. Growth plates of tibia and femur were scanned using a Zeiss Axiovert 35M microscope fitted with a LSR Astro Cam type TE3/A/S digital camera. The images were analyzed using Concord software from Life Science Resources Ltd.

3.5 ELECTROPHYSIOLOGY

3.5.1 Paired-pulse facilitation (PPF) recordings of hippocampal slices

Paper I: PN12-16 mice were terminally anesthetized with 100% CO_2 and sacrificed by decapitation. Brains were placed in ice-cold low Ca^{2+}/high Mg^{2+} artificial cerebrospinal fluid (aCSF) containing (in mM): 124 NaCl, 5 KCl, 1.24 NaH_2PO_4, 0.5 $CaCl_2$, 10 $MgSO_4$, 26 $NaHCO_3$, and 10 glucose, and oxygenated with 95% O_2/5% CO_2 (pH 7.4). Coronal hippocampal slices, 350 µm thick, were placed in regular aCSF (same composition as above but with 2.4 mM $CaCl_2$ and 1.3 mM $MgSO_4$). Patch-clamp electrodes (6-10 MΩ) were filled with (in mM): 135 Cs-methane sulphonate, 10 HEPES, 1 EGTA, 4 Mg-ATP, 0.3 Na-GTP, 2-5 QX-314 and 8 NaCl (pH 7.25; osmolarity 270-280 mOsm). Whole-cell voltage clamp (-70 mV) recordings of evoked excitatory postsynaptic currents (EPSCs) were obtained at RT from the soma of CA1 pyramidal neurons of the hippocampus, visualized with differential interference microscopy (DIC) using an Olympus BX50WI microscope. 50 µM picrotoxin (Sigma-Aldrich) was added to block $GABA_A$ receptor-mediated transmission. EPSCs were evoked by stimulation of Schaffer collaterals with fine concentric Pt/Ir bipolar stimulation electrodes (Fredrik Haer & Co) placed in the stratum radiatum. DC square

current pulses were delivered through the electrode at minimal intensity in order to activate as few presynaptic fibers as possible but yet achieve stable responses.

3.5.2 mEPP recordings from myotubes in co-culture

Paper II: Postsynaptic activities were electrophysiologically studied by a conventional intracellular recording method with sharp microelectrodes filled with 1 M potassium citrate (5-20 MΩ). Recording medium was 10 mM HEPES-buffered DMEM supplemented with 2 mM $CaCl_2$ and 0.1 mM choline chloride. Membrane potentials of myotubes were amplified via an Axoclamp 2A amplifier (Axon Instruments). Membrane potentials of DC-coupled or high gain RC-coupled recordings were monitored on a Nihon Koden thermal array recorder (model RTA-1100). The presence of miniature endplate potentials (mEPPs) in a given myotube that showed a synaptic-like connection to NG108-15 cells under a phase microscopy was judged by monitoring waveform on a storage oscilloscope.

3.5.3 Capacitance measurements in single primary beta cells

Paper III: Beta cells from NMRI mice showing GFP overexpression were used for whole cell patch clamp capacitance recordings of exocytosis. Cells were clamped at –70 mV to avoid activation of voltage-dependent Ca^{2+}-channels. Pipettes were pulled from borosilicate glass capillaries and coated with Sylgaard at their tips. The pipette resistance was 2-4 MΩ. The extracellular solution was composed of (in mM): 138 NaCl, 5.6 KCl, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 5 HEPES (pH 7.4) and 5 D-glucose. The pipette solution consisted of (in mM): 125 potassium glutamate, 10 KCl, 10 NaCl, 1 $MgCl_2$, 5 HEPES, 3 Mg-ATP, 10 EGTA, 7 $CaCl_2$ (pH 7.15). The free Ca^{2+}-concentration of the resulting buffer was 500 nM. Recordings were made using an EPC-9 patch clamp amplifier and the Pulse software (v. 8.35, HEKA Elektronik) at 33ºC.

3.5.4 hGH release assay

Paper III: INS-1E cells co-transfected with human growth hormone (hGH) and the plasmid of interest were incubated for 2 h in glucose-free medium and preincubated for 30 min in glucose-free Krebs-Ringer bicarbonate HEPES buffer (KRBH) consisting of (in mM): 135 NaCl, 3.6 KCl, 5 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 1.5 $CaCl_2$, 10 HEPES and 0.1% BSA (pH 7.4). Following preincubation, cells were incubated for 30 min in KRBH in the presence of 3 or 10 mM glucose. The supernatant was centrifuged at 100 *x g* and hGH in the resulting supernatant is referred to as secreted hGH. Cells were resuspended in 1 mM EDTA in PBS, transferred to the remaining pellet and lyzed by six freeze-thaw cycles. Insoluble material was pelleted and hGH in the supernatant is referred to as cellular hGH. hGH levels were measured using ELISA (Roche Diagnostics).

3.6 MOUSE BEHAVIOR ANALYSIS

Elevated plus-maze test. A plus-shaped maze made of gray Plexiglas was used, with two opposite enclosed and two open arms (each arm 30×5 cm) connected by a central area (5×5 cm) elevated 1 m above the floor (TSE, Bad Homburg). Each mouse was placed in the central area facing an open arm and was allowed to explore the maze for 5

min. Behavioral responses, recorded manually, were: the time spent in open arms and in closed arms and the number of crossings between closed and opened arms.

The Morris water maze test. Animals were handled by the operator for five days prior to the test. Spatial learning and memory were examined using a circular water tank (180 cm in diameter and 45 cm in height), surrounded by several extra-maze cues. The tank was filled with tap water (22-23ºC) mixed with Latex®. The escape platform, 15 cm in diameter, was placed in the south-east quadrant of the pool, 1 cm below the water level. The behavior of the animals in the water maze was monitored by a CCD camera (JVC) located above the water tank connected to the WaterMaze Software. For pre-training, the animals were tested 48 h prior to the spatial learning task (four trials) with a visible platform and a random selection of starting points in the absence of external cues. During training the hidden escape platform was located in the north-east quadrant. The mice were trained for five consecutive days (four trials per day with 30 min between each trial). The maximum trial duration was 90 s with each mouse spending 10 s on the platform at the end. During each trial, the mouse escape latency, swim speed and swim length were recorded.

Locomotor activity test. Motor activity was recorded by means of a multi-cage red- and infrared-sensitive motion detection system. The animals were habituated to the experimental room for at least 60 min prior to the experiment and they were individually tested in Macrolon locomotor cages for 60 min. The cages were equipped with horizontal and vertical photocell detectors. Motility was defined as all movements of a distance of 4 cm or more detected by 48 vertical photocells, and represents general activity. Locomotion was measured by counting the number of times an animal covered eight horizontal photocells or moved from one side of the cage to the other (at least 32 cm).

3.7 STATISTICAL ANALYSES

Student´s unpaired t-test was used for comparisons between mean values. In Paper III secretion data comparing multiple values was evaluated using Dunnett's test. A 95% confidence interval was used for determining levels relative to wt for mRNA and protein analyses in Paper I. Different kinds of variance analyses were used for statistical analysis of curves. Specifically, in Paper I growth curves and PPF ratios were compared with two-way repeated measurements analysis of variance (ANOVA). Data from the behavioral testing was analyzed by non-parametric statistics using Kruskal-Wallis ANOVA followed by Mann-Whitney U as post-hoc test. For electrophysiological measurements in Paper II, homogeneity of variances was tested with Fisher's F test followed by Student's t test using two-way analysis of homogeneous variance. All values are presented as means ± S.E.M., except for Dcx in paper I (S.D.).

4 RESULTS AND DISCUSSION

4.1 PAPER I: GENE TARGETED MOUSE MUTANTS FOR SNAP-25

During early neuronal development exclusively SNAP-25a is expressed but in most brain areas SNAP-25b becomes by far the major splice variant by the second postnatal (PN) week, at a time when SNAP-25 expression increases dramatically (Bark et al., 1995). Hence, in adult brain SNAP-25b has a prominent role in supporting regulated exocytosis. *In vitro*, SNAP-25b containing SNARE complexes are more stable than those formed with SNAP-25a and SNAP-25b has been demonstrated to enable a larger exocytotic burst (Sørensen et al., 2003; Sutton et al., 1998). We have now addressed what this translates into for neuronal function under *in vivo* conditions. We have developed SNAP-25b knock-out (KO)/SNAP-25a knock-in mouse mutants to evaluate the significance of the alternatively spliced isoforms of SNAP-25. The expression of SNAP-25b was replaced by supplementary SNAP-25a to retain physiological levels of SNAP-25 and thus specifically study the importance of the SNAP-25b isoform.

4.1.1 Determination of expression levels of SNAP-25 and developmental phenotype

Previously, a first attempt to investigate the functional importance of SNAP-25 variants by interfering with the normal SNAP-25a/SNAP-25b splicing ratio resulted in changes of synaptic plasticity and most of the homozygous mutants died during young adulthood. In addition, it was discovered that the presence of a neomycin resistance gene in the targeted SNAP-25 allele interfered with the expression of SNAP-25 (Bark et al., 2004). In the mouse mutants described here, analysis of mRNA and protein levels around PN14 revealed that before removal of the antibiotic selection gene SNAP-25 expression was repressed. These mice were termed unfloxed SNAP-25b mouse mutants (Figure 1). The reduction in expression was larger on the mRNA level compared to wt, suggesting that changes in protein levels are compensated for, for instance by increased protein stability. After *in vivo* floxing of the selection gene, using the CreLox system, SNAP-25 expression levels were restored to wt SNAP-25 levels and in addition slightly elevated. These mice were termed floxed SNAP-25b mouse mutants (Figure 2). Elevated SNAP-25a levels might be an attempt to compensate for the lack of SNAP-25b. No differences were detected in protein levels of the SNAP-25 homolog SNAP-23 and the binding partner Syntaxin 1. Relative brain mRNAs levels of SNAP-25a and SNAP-25b in wt at PN14 were 1:5 and homozygous mutants showed only SNAP-25a expression.

Unfloxed SNAP-25b null mouse mutants exhibited developmental defects (Figure 1F and G). Mutants were smaller in size, showed reduced bone growth and delayed eye opening and acquiring of hearing compared to wt littermates. At the end of the second PN week unfloxed mutants developed frequent episodes with hyperactivity and seizures and were sacrificed in the third PN week. Hence, comparisons between unfloxed and floxed mice were restricted to around PN14. Floxed mutants displayed neither of the phenotypic traits of the unfloxed mutants during early PN weeks except for that females exhibited reduced weight gain (Supplementary Figure 1). This suggests that levels of SNAP-25 protein are also important during development. It could be specific to early development when only SNAP-25a is expressed and at low levels, or

could become important when SNAP-25 expression rises and the switch to SNAP-25b occurs. In heterozygous SNAP-25 null mutants where expression is reduced but SNAP-25b is expressed no such developmental defects are detected. Therefore, the increase in SNAP-25 expression and/or the switch to SNAP-25b during postnatal development when synaptic activity becomes important for neuronal development must be necessary (Goodman & Shatz, 1993; Katz & Shatz, 1996). The Coloboma mouse has a spontaneous deletion encompassing the *Snap* gene (Hess et al., 1992). Heterozygous Coloboma mice show similar developmental defects such as hyperactivity as our unfloxed mutants despite retained SNAP-25b expression. However, there is several genes deleted in the Coloboma mice and this could represent features that are especially vulnerable during development, pointing to that polygenecity or additional factors such as malnutrition/stress may be required for neurological deficits to develop (Hess et al., 1994). The *Tkneo/Tkneo* SNAP-25 mouse model, where the neomycin resistance gene disturbed the alternative splicing, has reduced levels of total SNAP-25 and SNAP-25a/SNAP-25b ratio shifted towards more SNAP-25a expression (Bark et al., 2004). These mutant mice also exhibit spontaneous seizures and increased lethality, with a median survival time of 27 days. Hence, the lack of SNAP-25b appears more detrimental than reducing the total levels of SNAP-25 protein. If both the balance and the levels are altered, a severe developmental phenotype is observed.

Taken together, all the different SNAP-25 mouse models provide clues to the significance of SNAP-25 during neuronal development as well as in the mature nervous system. Our model however, is the first with only SNAP-25a expression and it specifically addresses the contribution of the two isoforms for SNAP-25 function. The differences in phenotype between unfloxed and floxed SNAP-25b mutant mice allows for additional comparison and evaluation of SNAP-25a and SNAP-25b during development and in adult.

4.1.2 Plasma membrane association of SNAP-25a and SNAP-25b and hippocampal short-term plasticity

The alternative splicing of SNAP-25 mRNA is an obligate choice between two exon 5s. Nine of 206 amino acids differ between SNAP-25a and SNAP-25b in the central domain of the protein where a quartet of cysteine residues are substrates for post-translational palmitoylation which is required for membrane targeting (Gonzalo et al., 1999; Lane & Liu, 1997). All four cysteines are retained in both SNAP-25a and SNAP-25b but appear in different organization and sequence context. We were interested in whether SNAP-25a and SNAP-25b showed a difference in association with the plasma membrane, which has been difficult to address due to that the similarities between the isoforms have prevented development of specific antibodies. As adult wt mice express essentially only SNAP-25b in brain (Bark et al., 1995) and our mutants only express SNAP-25a this allows for comparisons of subcellular localization of the SNAP-25 proteins. In sucrose density gradients, SNAP-25a was less associated with plasma membrane fractions than SNAP-25b and appeared to be more cytosolic or associated with synaptic vesicles (Figure 2E). This implies that SNAP-25a is less associated with SNARE complexes close to or immediate upstream of fusion than SNAP-25b. Similarly, tagged SNAP-25 proteins overexpressed in a glioma neuroblastoma cell line displayed a difference in subcellular distribution (Andersson et al., 2000). Mutational analysis of SNAP-23, a SNAP-25 homolog that resembles a combination of SNAP-25a

and SNAP-25b in terms of cystein residue localization, showed that it is the conserved residues that are required for membrane localization (Koticha et al., 1999). The number of palmitoylated residues might confer a difference in plasma membrane preference as SNAP-23 shows a higher degree of lipid raft association than SNAP-25 in PC12 cells (Salaun et al., 2005). The different SNAP-25 isoforms have not been investigated in raft association.

Short-term plasticity reflects the properties of a synapse to adapt to activity-based changes without long-lasting effects such as gene expression (Zucker & Regehr, 2002). Presynaptic short-term plasticity can be measured in paired-pulse facilitation (PPF) experiments. Two pulses, generating action potentials, are applied with a short interval so that residual Ca^{2+} in the presynaptic terminal after the first pulse results in a higher Ca^{2+}-concentration after the second (Charlton et al., 1982; Katz & Miledi, 1968). This process is faster than for mobilization of vesicles to refill the RRP. Thus only remaining fusion-competent vesicles requiring a higher Ca^{2+} concentration are released and facilitation then reflects the size of the RRP. We investigated PPF of AMPA receptor-mediated synaptic transmission at the Schaffer collateral-CA1 pyramidal neuron synapses of the hippocampus. We analyzed PN12-16 animals for comparison of unfloxed and floxed SNAP-25b KOs. Both mutants showed a reduction in PPF compared to wt mice at low frequency stimulation and unfloxed animals also at higher frequency stimulation (Figure 3A,B,C). Neither in the *Tkneo/Tkneo* nor in the Coloboma SNAP-25 mutant mice was reduced PPF detected in the hippocampal formation; instead the *Tkneo/Tkneo* mutants demonstrated an increase in PPF. This suggests that reduced SNAP-25 protein levels do not affect hippocampal PPF as long as SNAP-25b is present (Bark et al., 2004; Steffensen et al., 1996). The reduction in facilitation indicates a reduced stability and size of the RRP. The synaptic active zone holds few ready-releasable vesicles and is dependent on replenishment of the RRP, which is regulated by synaptic activity (Stevens & Wesseling, 1998). PKA and PKC phosphorylation of the SNAP-25 protein both enhance the releasable pool sizes but specifically PKC increased binding to Syntaxin 1 and replenishment of the RRP in chromaffin cells (Nagy et al., 2002; Nagy et al., 2004; Yang et al., 2007). The Rab3 effector Rabphilin interacts with SNAP-25 and potentially regulates the refilling of vesicle pools after activity (Deak et al., 2006; Tsuboi et al., 2007). Changed short-term plasticity can also have other causes than replenishment properties. For instance, the size of the RRP has been inversely correlated with release probability of vesicles (Dobrunz & Stevens, 1997; Murthy et al., 1997). Thus a reduction in facilitation, signifying a smaller RRP, might be due to a general increase in release probability of vesicles. The size of the vesicle pools and the probability of release should be measured in our mutants to further establish plasticity changes.

In embryonic SNAP-25 null chromaffin cells introduction of SNAP-25b showed higher ability to keep vesicles in the primed state than SNAP-25a, not due to enhanced priming but rather dependent on a lower de-priming rate (Sørensen et al., 2003). SNAP-25b overexpression increased the size of the releasable pools, demonstrated by an increased exocytotic burst (Sørensen et al., 2003). It has been demonstrated that SNAP-25b containing SNARE complexes are more stable than those having SNAP-25a (Sutton et al., 1998), suggesting that SNAP-25b adds constraint to the fusion reaction by requiring a higher stimuli prior to fusion. The blind-drunk mouse, with a mutation in SNAP-25b increasing SNARE complex stability even further, instead showed reduced spontaneous and evoked release (Jeans

et al., 2007). This was proposed to be due to abnormalities to replenish the RRP, which could result from impaired disassembly of the SNARE complexes. The higher stability of the SNARE complexes in the presence of SNAP-25b may besides increasing the RRP also allow for differences in affinity of a SNARE complex binding protein. The difference in exocytotic burst capacity between SNAP-25a and SNAP-25b was shown to be due to amino acids located in the N-terminal helix and was suggested to regulate association with other proteins during the formation of the SNARE complex (Nagy et al., 2005). Complexin does not bind directly to SNAP-25 but to Syntaxin 1 and VAMP2 in the assembled SNARE complex and performs a yet unidentified function, aiding the interaction with Synaptotagmin I (Melia, 2007). Complexin appears to bind with different affinities depending on the state of assembly of the SNARE complexes and thus is a candidate for differential SNAP-25 actions (Liu et al., 2006). Complexin I and II deficient mice exhibit no change in pool sizes in neurons but a reduced probability of release reflected by increased facilitation (Reim et al., 2001). Furthermore, specifically synchronous release is affected which may indicate impaired Synaptotagmin I function. The vesicle-associated SNAP-25 binding protein Snapin is proposed to stabilize the pool of primed vesicles by increasing the association of SNARE complexes with Synaptotagmin I as demonstrated in Snapin-deficient chromaffin cells (Tian et al., 2005). Here a potential difference for the SNAP-25 isoforms can be implicated, as the pool of primed vesicles is more unstable in SNAP-25a containing neuroendocrine cells than in neurons, with preferentially SNAP-25b (Heidelberger et al., 2002; Xu et al., 1998). In neurons, with a small size of the RRP, Snapin appears not essential but merely to enhance release probability and regulate plasticity when phosphorylated by PKA (Thakur et al., 2004). Besides interacting with the SNARE complex Synaptotagmin I may also bind to Syntaxin 1/SNAP-25 and all three proteins interact with Ca^{2+}-channels (Rettig et al., 1996; Sheng et al., 1994). This may regulate channel properties but also provide crucial proximity between Ca^{2+}-channels and the release machinery (Evans & Zamponi, 2006). Furthermore, the active zone component RIM performs several actions during exocytosis and one of them involves binding to SNAP-25, Synaptotagmin I and Ca^{2+}-channels (Coppola et al., 2001). In the presence of Ca^{2+} the interaction with Synaptotagmin I of RIM was demonstrated to be increased whereas the binding to SNAP-25 was reduced. SNAP-25 may also be subject to G protein coupled inhibition, as Gβγ binds to the C-terminal of SNAP-25, both in central synapses and in neuroendocrine PC12 cells (Blackmer et al., 2005; Gerachshenko et al., 2005). This interaction is inhibited by binding of Synaptotagmin I to SNAP-25 at the C-terminal (Blackmer et al., 2005; Gerona et al., 2000). Hence, there are several links between SNAP-25 and the presumed Ca^{2+}-sensor Synaptotagmin I, and preference of different Synaptotagmin isoforms themselves may also differ for SNAP-25a or SNAP-25b containing SNARE complexes. The differences in affinity for proteins, including their isoform variants, binding to SNAP-25a or SNAP-25b SNARE complexes have so far been little studied.

The notion that spontaneous transmitter release is not affected in SNAP-25 null mutants suggests that a SNAP-25 homolog can replace early developmental activities of SNAP-25 (Molnar et al., 2002; Washbourne et al., 2002). Spontaneous release occurs in the presence of basal Ca^{2+}-concentrations, appears not to involve Synaptotagmin I and can be performed by SNAP-23 (Chieregatti et al., 2004; Geppert et al., 1994). Overexpression of the ubiquitously expressed SNAP-23 in SNAP-25 null

chromaffin cells could partly rescue sustained secretion but the exocytotic burst component was impaired, implying SNAP-23 in slower modes of exocytosis (Sørensen et al., 2003). In brain for instance glia cells express SNAP-23 and it could be involved in GABAergic inhibitory transmission though this has not been clarified (Montana et al., 2006; Tafoya et al., 2006; Verderio et al., 2004). A novel SNAP-25 homolog termed SNAP-47 was recently reported, also showing ubiquitous expression and a lower ability to support exocytosis than SNAP-25, with a proposed intracellular function (Holt et al., 2006). Intriguingly, the SNAP-25 homolog SNAP-29, which lacks a membrane anchor, appears to regulate SNARE complex disassembly in hippocampal neurons, slowing recycling and recovery after exocytosis events (Pan et al., 2005; Steegmaier et al., 1998). During high-frequency stimulation and short interpulse intervals, the reduction of PPF was not significant in floxed SNAP-25b null mice (Figure 3D). Thus, at physiological levels of SNAP-25 expression and more sustained stimulation, SNAP-25a was as efficient as SNAP-25b in supporting release. Apparently increased Ca^{2+}-concentrations favor the priming process of SNAP-25a dependent vesicles. Increased Ca^{2+}-concentrations also overcome the release phenotype in ComplexinI/II mutants (Reim et al., 2001).

Thus, SNAP-25b is required to meet the demands of synaptic transmission including maintaining vesicle pools ready for release at the Schaffer collateral-CA1 synapse. SNAP-25a appears better adapted to slower release from LDCVs, being the major isoform in neuroendocrine and endocrine cells. The difference between the two SNAP-25 isoforms could be illustrated as SNAP-25b tighter holding the vesicles in a highly release-ready state whereas SNAP-25a provides a looser arrangement, which might affect interactions with proteins regulating release via the SNARE complex. SNAP-25a would then be closer related to other identified SNAP-25 homologs than SNAP-25b. Early synaptic development seems not to be highly dependent on neuronal activity, at a time when only SNAP-25a is expressed (Goda & Davis, 2003). However, during the first PN weeks functional neuronal circuits are developing and the emergence of mature neuronal connections requires preferential signaling from active synapses, while others with low activity are eliminated. PPF has been demonstrated to peak around PN14 (Dekay et al., 2006; Muller et al., 1989) and reduced PPF in our mutants could result in that the SNAP-25b KO mutants fail to develop accurate neuronal circuits. Possibly unfloxed mutants experience additional impairments in neuronal development that is reflected by lower short-term plasticity than in floxed mutants.

4.1.3 Behavior analyses of floxed SNAP-25b null mice demonstrate cognitive impairment

SNAP-25b is normally highly expressed in brain areas involved in cognitive function (Bark et al., 1995; Oyler et al., 1989), and behavior testing of four months old floxed SNAP-25b null mutants revealed broad cognitive impairment. The mutants exhibited increased anxiety levels and almost complete absence of acquisition and retention of spatial learning (Figure 4). Furthermore, mutants had low context-dependent locomotor activity, suggesting less exploratory behavior (data not shown). All in all, our mutants display a phenotype with impairments in hippocampus and amygdala. As motor neurons reportedly only express SNAP-25b (Jacobsson et al., 1996), it was of interest to see how the mutants handled muscle endurance tasks. The rotorod test of

motor coordination and muscle strength was however cancelled due the bad condition of the mutants after the Morris swim test of spatial learning, but preliminary results showed signs of muscle weakness. The worse performance in the spatial learning test and the lower locomotor behavior during initial exploration of the locomotor cages of females, together with that some female mutants were lost during behavioral testing; all suggest that females were in worse condition than males. Whether this depends on developmental defects, as floxed females were smaller than wts during the second PN week, or different sensitivity to alterations in synaptic plasticity we currently do not know. Besides its well-known hypothalamic actions estrogen exerts effects on hippocampal synapses (McEwen, 2002). Estrogen apparently directly influences inhibitory presynapses and postsynaptic dendritic spines are sensitive to sex hormones, which could potentially render our females more vulnerable.

The hyperactivity of young unfloxed mutants was not observed in floxed animals and presumably does not develop in the absence of developmental defects. Hyperactivity is detected in the Coloboma mouse, which shows developmental defects, and is considered a model of attention deficit hyperactivity disorder, ADHD (Hess et al., 1992). Despite that several genes are deleted in the Coloboma mouse hyperactivity is abolished when reintroducing SNAP-25b and together with that ADHD often improves with age, this implicates the importance of accurate neurotransmission during development. In particular, dopamine and norepinephrine release is impaired in these mice (Jones & Hess, 2003; Raber et al., 1997). Also, corticosterone levels in response to stress were increased in Coloboma mice, which could contribute to impairments in behavior (Raber et al., 1997). In humans, polymorphisms in the *Snap* gene has been associated with vulnerability to develop ADHD and interestingly, with intelligence (Faraone et al., 2005; Gosso et al., 2006; Thapar et al., 2005). Imbalances in release of substances such as dopamine and norepinephrine need to be addressed in both unfloxed and floxed SNAP-25b KO mice as the importance of the isoforms has not been investigated. The blind-drunk mouse with a mutation in SNAP-25b and increased SNARE complex affinity exhibits ataxia and impaired sensorimotor gating (Jeans et al., 2007). These are behavior problems associated with psychiatric disorders such as schizophrenia, to which SNAP-25 has been linked (Mirnics et al., 2001). The SNARE complex binding Complexins have also been associated with a number of psychiatric diseases (Eastwood 2000). Complexin I and II double KO mice die at birth (Reim et al., 2001). However, Complexin I or II deficient mice share some behavioral abnormalities with the floxed SNAP-25b mutants, such as deficits in motor function, spatial learning and exploratory behavior (Glynn et al., 2003; Glynn et al., 2005). Interestingly, cognitive behavior deficits develop with age, as might be the case in our floxed mutants. Complexins are linked to the degenerative Huntington´s disease and Complexin II deficient mice show specifically impairment in mossy fiber long-term plasticity (Gibson et al., 2005; Morton & Edwardson, 2001). Thus, accuracy in the last steps of exocytosis after SNARE complex assembly appears important for long-term neuronal function. Long-term plasticity is required for memory and learning and involves structural rearrangements and gene expression and would be interesting to investigate both in Schaffer collaterals and mossy fibers in the SNAP-25b KO mice. Interestingly, both SNAP-25a and SNAP-25b were upregulated after long-term potentiation induction, a form of long-term plasticity, and thus SNAP-25a could be

involved in such rearrangements in the adult brain (Hou et al., 2004; Hou et al., 2006; Roberts et al., 1998).

4.1.4 Hippocampal neuroprotective mechanisms in response to seizure activity and morphological changes

During the high activity of synaptic maturation at PN weeks two and three, the brain is particularly vulnerable to develop seizures (Swann & Brady, 1984). Altered synaptic activity during this time point might initiate spontaneous seizures, which both unfloxed and floxed SNAP-25b null mutants demonstrate. The unfloxed mutants are most severely affected with frequently occurring seizures debuting around PN12-13, whereas in floxed mutants seizure activity is rare prior to young adulthood. The developmental phenotype of the unfloxed mutants as well as the distorted short-term plasticity could contribute to the severe seizure activity. Epileptic seizures are characterized by high neuronal activity eventually leading to brain damage (Wasterlain et al., 2002). Even though seizures do not necessarily origin in the hippocampus, recurrent seizure activity can be manifested in this area because of its ability to adapt to changes in activity. It is particularly the naturally excitable CA3 neurons that may contribute to development of recurring seizure activity (Congar et al., 2000; Scharfman et al., 2000).

Neuropeptides are involved in modulating a number of physiological functions in brain such as energy balance, feeding behavior, mood, memory and learning (Hebb et al., 2005; Vezzani & Sperk, 2004). The neurotrophin Brain-derived neurotrophic factor (BDNF) has besides developmental effects also functions in the adult synapse, such as mediating activity-dependent actions involved in memory and learning (Lu, 2003). Typically expression of neuropeptides and BDNF is altered in the hippocampus after seizures, in particular in the granule cell-mossy fiber system, and is considered to be a neuroprotective mechanism (Gall et al., 1990; Schwarzer et al., 1996; Wasterlain et al., 2002). We observed downregulation of Cholecystokinin (CCK) expression and upregulation of Neuropeptide Y (NPY) and BDNF expression in the hippocampus of our mutants after seizure onset (Figure 6). Increased NPY expression is believed to be neuroprotective by dampening excitatory activity and BDNF can regulate NPY expression (Koyama & Ikegaya, 2005; Vezzani & Sperk, 2004). However, what effects long-term changes in expression have is not certain. BDNF overexpressing mice for instance, are susceptible to seizures (Croll et al., 1999). BDNF signaling increases synaptic protein levels and phosphorylation states and the number of docked vesicles (Lu, 2003; Tyler et al., 2002). Furthermore, the same neuropeptide may exert both excitatory and inhibitory modulation through binding to different receptors (Hebb et al., 2005; Vezzani & Sperk, 2004). In the end, neuroprotective attempts might not always be beneficial to highly plastic areas of the brain.

In the hippocampus neurogenesis frequently increases in rodents after seizures, which we also detected in our mutants (Supplementary Information). Accordingly, neuronal activity can induce neurogenesis (Deisseroth et al., 2004). In animals displaying seizures however, the subgranular layer of neuronal precursor cells frequently appears dispersed and ectopically located new neurons might contribute to CA3 hyperexcitability (Jessberger et al., 2005; Parent et al., 1997). Though, it has been shown that newly generated neurons after epilepsy exhibit dampening characteristics (Jakubs et al., 2006; Scharfman et al., 2000). As both NPY and BDNF have been suggested to promote neurogenesis their protective effects may also be mediated in this

way (Howell et al., 2005; Scharfman et al., 2005). Immunohistochemical analysis revealed that from two months of age mossy fibers in floxed mutants appeared expanded and bundled together (Figure 5,6). The Synaptophysin positive nerve endings appeared larger but less numerous, suggesting a locally decreased density of functional nerve endings. The mossy fiber-CA3 synapses are highly plastic and can increase in size in response to altered activity (Galimberti et al., 2006). Hence, the morphological changes may be an adaptation to increased activity, supported possibly by increased neurogenesis. Although altered synaptic transmission as well as seizure activity can impair cognitive functions (Sutula et al., 1995), it should not be excluded that the morphological changes per se highly contribute to behavioral impairment in our mutants. Besides being generally resistant to seizure development, seizures do not seem to affect performance in spatial learning of C57BL/6 mice (Royle et al., 1999). It has been demonstrated that this mouse strain is fairly resistant to seizure-induced hippocampal cell death and we could not observe any loss of cell layers in mutants with histology, which might have had contributed to cognitive impairment.

Thus, the plastic hippocampus may adapt to increased activity by trying to increase its excitatory routes such as the mossy fiber system. In fact, seizures have been referred to as an extreme form of plasticity that may have gone too far (Scharfman, 2002). The combination of altered synaptic transmission during neuronal development and subsequent seizure activity results in changes in plastic brain areas, with accompanying cognitive dysfunction and brain damage.

4.2 PAPER II: CDK5 IN DEVELOPMENT OF FUNCTIONAL SYNAPSES

Cdk5 plays a major role in neuronal migration during neuronal positioning and differentiation (Dhavan & Tsai, 2001; Nikolic et al., 1996; Paglini et al., 1998). Gene targeted removal of Cdk5 in mice leads to widespread disruption of neuronal layering and neonatal death (Ohshima et al., 1996). Disruption of the p35 gene results in aberrant layering of the neocortex (Chae et al., 1997). Gene targeting of the p39 gene does not give an obvious phenotype, however the double p35/p39 KO resembles Cdk5 KO mice (Ko et al., 2001), indicating that p35 and p39 are the only activators of Cdk5. The impaired neuronal positioning found in the Cdk5 and p35 KOs could partly be caused by deregulated axon guidance. Cdk5 transduces cell surface-derived signals in several intracellular pathways, resulting in cytoskeletal rearrangements. For example, Cdk5 phosphorylation of β-catenin disrupts N-cadherin mediated cell adhesion thereby promoting neuronal migration (Kwon et al., 2000). Cdk5 has also been implicated in Integrin and Netrin 1 signaling (Del Rio et al., 2004; Ledda et al., 2002). Cdk5 is involved in growth cone collapse during axon guidance and positioning, for instance through phosphorylation of Collapsin response mediator protein-2 (CRMP-2) (Brown et al., 2004; Gillardon et al., 2005), and Cdk5 inhibits Pak1 (Nikolic et al., 1998; Rashid et al., 2001). Thus, in the presynaptic terminal a number of actin modulating proteins are Cdk5 substrates. Cdk5 influences cytoskeletal stability through phosphorylation of Neurofilaments and Microtubule-associated proteins (MAPs) (Kesavapany et al., 2004; Shea et al., 2004; Smith, 2003). In neurons p35 has been far more studied than p39 and so far only Tau, normally functioning in tubulin plasticity, has been shown to require p39 (Takahashi et al., 2003). Hyperphosphorylation of Tau by Cdk5 is implicated in several degenerative diseases, but then interestingly with the more stable Ca^{2+}-dependent calpain cleavage product of p35, p25 (Patrick et al., 1999). In addition, Cdk5 phosphorylates a number of transcription factors important both in differentiation of neuronal and non-neuronal cells (Rosales & Lee, 2006).

To investigate if Cdk5 is involved in the establishment of functional synapses and SV exocytosis after outgrowth, we used the mouse rat neuroblastoma-glioma cell line NG108-15. Upon dBcAMP-induced differentiation NG108-15 cells acquire a neuron-like phenotype, express for example N-type Ca^{2+}-channels, and can be stimulated to release ACh (Higashida, 1988; Kasai & Neher, 1992; Tojima & Ito, 2004). NG108-15 cells do not form functional autaptic synapses but when co-cultured with differentiating muscle cells functional d-tubocurarine-sensitive cholinergic synapses are formed (Higashida et al., 1981; Kimura & Higashida, 1992; Nelson et al., 1976). The presence of Cdk5 and both activator proteins p35 and p39 in the NG108-15 cell line was determined with RT-PCR, Western blotting and immunocytochemistry (Figure 1-5). Judging by Western blotting, levels of Cdk5 and p35 proteins increased with differentiation, as seen in other cells of neuronal origin (Fu et al., 2002; Munoz et al., 2000; Paglini et al., 1998; Tomizawa et al., 1996). p35 expression is upregulated by Nerve-growth factor (NGF) induced differentiation through the Extracellular-signal-regulated kinase (ERK) pathway (Harada et al., 2001). Cdk5 and p35 immunoreactivities were concentrated to low-density fractions where soluble proteins and proteins associated with light membranes distribute (Figure 2). Using synaptic proteins as markers for different subcellular compartments it was showed that in differentiated cells Cdk5 and p35 distribution overlapped with that of plasma

membrane and SVs. Levels of p39 remained low and its distribution overlapped mainly with Synaptotagmin I, suggesting its localization to LDCVs or other organelles of high density. Performing soluble/membrane or cytoskeletal fractionations revealed that while Cdk5 predominantly localized to soluble fractions, p35 had a higher degree of localization to membranes and p39 was almost exclusively associated with membrane and cytoskeletal structures (Figure 3). Interestingly, in NG108-15 cells, despite having a myriostylation site as does p39 (Patrick et al., 1998; Patzke & Tsai, 2002), p35 was preferentially located to cytosolic fractions. This could be an artefact of a cell line but also reflect that most p35 is not preferentially associated with structures but instead cytosolic. Since most identified Cdk5 substrates are either membrane- or cytoskeletal-associated proteins, or even cytoskeletal components themselves (Smith & Tsai, 2002), it is possible that the detergent-insoluble pool of the Cdk5 activators is in close proximity to, or even interacting with, potential substrate proteins (Floyd et al., 2001; Humbert et al., 2000a; Nikolic et al., 1998; Nikolic et al., 1996). Hence, Cdk5 activity might be spatially directed by the localization of the activators. In brain, the temporal and spatial expression of p35 and p39 seem to be complementary, with p39 expression initiated later in development (Cai et al., 1997; Wu et al., 2000; Zheng et al., 1998). During development p39 expression is prominent in brain but in adulthood that remains only in certain structures of the cerebellum (Jeong et al., 2003). The activators appear to localize to distinct subcellular compartments, with some overlap, in growth cones and synapses (Humbert et al., 2000a; Humbert et al., 2000b). Interestingly, when transiently overexpressed in NG108-15 cells, most p35 protein accumulated in membrane and cytoskeletal fractions. Prior to use, p35 might be attached to insoluble components and after phosphorylation of the substrate the different components might be dissociated. Immunoreactivities of p35 and p39 were prominent in developing neurites compared to Cdk5, but were less plasma membrane localized than SNAP-25 (Figure 4,5 and data not shown). It is difficult to study proteins in NG108-15 cells in co-culture with myotubes, and levels and subcellular localization of the different proteins might very well be different from when NG108-15 cells are cultured alone. Immunocytochemical comparisons however showed no obvious differences in for instance plasma membrane association.

To assess the impact of Cdk5 and its regulatory subunits on synaptic activities, postsynaptic membrane potential fluctuations, due to spontaneous quantal transmitter release, were recorded from myotubes co-cultured with transiently transfected NG108-15 cells. By measuring miniature endplate potentials (mEPPs) in postsynaptic cells demonstrating cell-cell contacts resembling synaptic structures, it is possible to analyze the formation of functional synapses. The percentage of synaptic-like structures exhibiting spontaneous quantal release day 5-7 in wtCdk5-transfected cells were not different from controls (~43%). However, with a dominant negative (dn) Cdk5 the level of active cell-cell contacts was decreased to 26%. By using NG108-15 cells stably overexpressing Synapsin IIa, (NG108IIA1), expected to exhibit a higher efficiency in forming active synaptic-like structures (Han et al., 1991; Zhong et al., 1999), dnCdk5 caused a similar reduction. An increase in frequency of postsynaptic currents is considered to denote an increase in release from presynaptic release sites. To investigate if spontaneous synaptic activity was altered as a result of transfection, frequency of mEPPs was registered and gave similar effects as numbers of synaptic-like structures. These results show that overexpression of dnCdk5 negatively affects functional synapse formation. The changes in mEPP frequency for formed synaptic

structures suggest that Cdk5 directly stimulates the exocytotic machinery. Considering the differences in protein levels detected by Western blotting, the levels of Cdk5 activators, and not Cdk5, could be limiting for kinase activity. This has been demonstrated in hNT cells where a substantial increase in Cdk5 activity is coupled to an increase in p35 expression (Gompel et al., 2004). Overexpression of either of the two activators, p35 or p39, showed a higher rate of functional synapse formation after four days in co-culture (Figure 7). The levels of synaptic structures exhibiting spontaneous release were similar in Cdk5/p35 and Cdk5/p39 overexpressing cells, 73% and 75%, compared to 43% for non-transfected cells. Both p35 or p39 also increased mEPP frequency, 5.8 and 8.4 events/min compared to ~4 in control, however p39 seemed more potent than p35. Thus, both p35 and p39 may mediate spontaneous release from SVs. However the low endogenous levels of p39 after differentiation suggest that p35 has a broader role in NG108-15 cells.

Transition of a growth cone into a presynaptic terminal requires, except for structural changes, assembly of an exocytotic machinery that can support vesicular fusion. In membrane transport Cdk5/p35 has been reported to play a role in the budding of vesicles from the Golgi apparatus (Paglini et al., 2001), and kinesin-driven motility is regulated by Cdk5 (Morfini et al., 2004). Synapsin I, a presynaptic protein involved in tethering of vesicles to the actin cytoskeleton and in plasticity, has been shown to be a Cdk5 substrate, although the functional implication of Cdk5 phosphorylation is not clear (Matsubara et al., 1996; Südhof, 2004). Synaptic transmission, including spontaneous neurotransmitter release, is abolished in neurons from Munc13 and Munc18-1 null mouse mutants, indicating the Cdk5 substrate Munc18-1 as a factor in mediating Cdk5 enhancement of release in our study (Varoqueaux et al., 2002; Verhage et al., 2000). In these mice initial presynaptic assembly in central neurons appeared normal in contrast to at the NMJ, where terminal growth of neurons was defect and synapse numbers were reduced (Brandon et al., 2003; Goda & Davis, 2003; Misgeld et al., 2002). Also, whereas spontaneous release at the NMJ was enhanced by Munc18-1 overexpression, it reduced the frequency of spontaneous release in central neurons (Ho et al., 2006; Toonen et al., 2006b). This demonstrates differences in dependence on transmission for development as well as in release mechanisms of these synapses. Also, Cdk5/p35 regulates synaptic vesicle endocytosis by phosphorylating three of the endocytotic dephosphin proteins, Dynamin 1, Amphiphysin 1 and Synaptojanin 1 (Lee et al., 2004b; Tan et al., 2003; Tomizawa et al., 2003). These proteins are activated by Calcineurin dephosphorylation and Cdk5 seems to maintain the resting state of these proteins. All these events may enhance the rate of synapse formation and spontaneous release in transfected NG108-15 cells. However, in evoked transmission the organization of the exocytotic machinery may differ to some extent as in the striatum dopaminergic and glutamatergic transmission appears negatively regulated by Cdk5 (Chergui et al., 2004). This may occur via p35-mediated phosphorylation of the P/Q Ca^{2+}-channel, thereby disrupting its interaction with SNAP-25 and Synaptotagmin I (Tomizawa et al., 2002; Yan et al., 2002).

The establishment of synapses at the NMJ displays a pattern of both independent processes in neurons and muscles and in addition involves reciprocal intersynaptic signaling (Goda & Davis, 2003). Agrin signaling supports clustering of postsynaptic ACh receptors where innervation has occurred (Lin et al., 2001; McMahan, 1990). The growth factor Neuregulin-1 signals via ErbB receptors and Cdk5/p35 appears to be operating in this pathway, increasing ACh receptor expression and associating with

ErbB receptors (Fu et al., 2001). In addition, Cdk5 was recently discovered to be involved in dispersion of ACh receptors at non-innervated postsynaptic sites (Fu et al., 2001; Fu et al., 2005). At the postsynaptic density of central neurons Cdk5 phosphorylates NMDA receptors and Post-synaptic density protein-95 (PSD-95) and controls CAMKII in a Ca^{2+}-dependent manner (Dhavan et al., 2002; Hosokawa et al., 2006; Morabito et al., 2004; Wang et al., 2003; Wei et al., 2005b). Furthermore, Cdk5 was recently demonstrated to be required for BDNF induced dendritic growth (Cheung et al., 2007). Cdk5 activity is thereby linked to long-term plasticity and neurotoxicity.

Thus, Cdk5 has an increasingly recognized pivotal role in both developing and mature neurons and synapses and we have demonstrated that in establishment of functional signaling, Cdk5 is promoting spontaneous synaptic activity via a presynaptic mechanism. Both activator proteins are capable of supporting this action of Cdk5 though the effect of p39 overexpression was larger.

4.3 PAPER III: CDK5 IN PANCREATIC BETA CELLS

Evidence has emerged showing that Cdk5 also has a function in non-neuronal tissues, for instance in muscle development and wound healing (Rosales & Lee, 2006). Our group has demonstrated that Cdk5 is endogenously expressed in pancreatic beta cells and is able to enhance insulin secretion (Lilja et al., 2001). To continue this line of research, we characterized the expression of the Cdk5 activators p35 and p39 and investigated their role in insulin secretion. RT-PCR, Western blotting and immunocytochemistry detected both Cdk5 activators p35 and p39 in primary beta cells and p39 appeared to be the most abundant activator by Western blotting (Figure 1,2). Subcellular fractionation showed that both activators were associated with intracellular membranes, although of different densities, in both unstimulated and glucose-stimulated beta cells (Figure 3). The activator p39 may be associated with secretory granules as it is to some extent co-localized with insulin in subcellular fractionation analyses. Both p35 and p39 showed partly co-distribution with a plasma membrane marker and some p35 was also detected in the potentially soluble fraction. The difference in localization of p35 in NG108-15 cells and beta cells suggest some differences between these cell types in terms of Cdk5 substrates present, not unlikely for such morphologically diverse cells. Hence, localization and expression of the short-lived activators are regulating Cdk5 activity (Patrick et al., 1998; Patzke & Tsai, 2002; Wei et al., 2005b). The difference in localization of Cdk5 and the activators suggest that the interactions are transient. It has been suggested that Cdk5 cycles between cellular compartments whereas the activators are stationary, associated with insoluble components, and help to target the kinase to its substrates (Cheng et al., 2002; Floyd et al., 2001; Nikolic et al., 1996).

The effects of p35 and p39 overexpression on insulin exocytosis in mouse beta cells were explored using capacitance measurements (Figure 4). This technique detects cell surface area expansion reflecting Ca^{2+}-dependent exocytosis. In cells transiently transfected with p39 the rate of increase in cell capacitance was accelerated by 83%, compared to mock transfected cells. Overexpression of p35 did not stimulate exocytosis. WtCdk5 stimulated exocytosis by 44% whereas the dnCdk5 mutant decreased the rate of capacitance increase with 33%. In order to investigate the role Cdk5 and its activators play in the regulation of insulin exocytosis, we transfected cells with antisense oligonucleotides. The rate of exocytosis was inhibited by 42% in cells

treated with Cdk5 antisense oligonucleotides. Antisense treatment of p39, but not p35, inhibited exocytosis to an extent similar to that of antisense Cdk5. To ensure that the effects of Cdk5 and p39 on capacitance measurements were due to stimulation of exocytosis and not influenced by endocytosis, we measured release of transfected human growth hormone (hGH) from INS-1E cells (Figure 5). Co-transfection with Cdk5 or p39, but not p35, enhanced glucose-induced hGH secretion by 112% or 122%, respectively, whereas basal release was not changed.

A role of Cdk5/p35 in beta cells was recently demonstrated in regulation of the *insulin* gene, implicated in glucotoxicity where p35 expression was upregulated (Ubeda et al., 2006). Furthermore, Cdk5/p35 was demonstrated to have a negative effect on high-glucose stimulated insulin secretion, both in primary beta cells and MIN6 cells (Wei et al., 2005a). The Cdk5/p35 phosphorylation of L-type Ca^{2+}-channels also interfered with Syntaxin and SNAP-25 binding in beta cells during glucose-stimulated secretion mediating a moderate decrease in Ca^{2+}-influx (Wei et al., 2005a). However, exactly how high-glucose conditions translate to physiological settings remains vague. Apparently inhibition of Cdk5 had no effect on Ca^{2+}-channels and secretion in the absence of glucose and thus suggests Cdk5/p35 involvement in either the second phase of insulin release or an amplifying pathway. This illustrates the complex regulation of insulin release in beta cells.

It is not unlikely that the multifunctional protein Cdk5 has a number of yet unidentified functions also in pancreatic beta cells and we have identified a role at a late step in insulin release. The actions of Cdk5 are most likely intimately coupled to the activator specific for each substrate. In terminally differentiated secretory cells Cdk5/p39 can directly affect the exocytotic machinery, though involvement in earlier steps cannot be excluded.

4.4 PAPER III: MUNC18-1 IN INSULIN SECRETION

In search for the mechanism by which Cdk5 enhances insulin secretion we focused on proteins that operate close to the fusion event. Munc18-1 has been demonstrated to be a PKC and Cdk5 phosphorylation substrate in other cell types, however, the role of Munc18-1 in beta cell exocytosis as well as the function of the Cdk5 phosphorylation is not characterized (Fletcher et al., 1999; Fujita et al., 1996; Shuang et al., 1998). We confirmed the presence of Munc18-1 in primary beta cells (Jacobsson et al., 1994; Zhang et al., 2000) by Western blotting and immunocytochemistry (Figure 2,3). To address the question if Cdk5/p39 could operate via phosphorylation of the Munc18-1 protein, we performed capacitance measurements on beta cells transiently transfected with Munc18-1 wt and non-phosphorylatable templates in combination with Cdk5 and the Cdk5 activators (Figure 6). Since the aim was to investigate if Cdk5 regulates Munc18-1 function we tried to avoid activation of other kinases by excluding cAMP in the patch pipette. To confirm that we did not activate PKC we included an expression construct where all potential PKC phosphorylation sites were silenced. Overexpression of Munc18-1 wt or the PKC phosphorylation mutant both had a small positive effect on capacitance increase, an effect that was absent with the Cdk5 phosphorylation mutant. Co-transfection of Munc18-1 with Cdk5 or p39 increased insulin exocytosis significantly, whereas co-transfection of p35 did not augment secretion. Hence, as detected in our experimental setup Munc18-1 has a stimulatory function in beta cell exocytosis, which is augmented by Cdk5/p39 phosphorylation.

Both PKC and Cdk5 phosphorylation of Munc18-1 reduce its affinity for Syntaxin 1 (Barclay et al., 2003; de Vries et al., 2000; Fletcher et al., 1999; Fujita et al., 1996; Gladycheva et al., 2004; Liu et al., 2004; Shuang et al., 1998). Hence, phosphorylation might mediate Munc18-1 dissociation from Syntaxin 1 for SNARE complex assembly as well as Munc18-1 functions not involving Syntaxin 1. However, crystallization of Munc18-1 in complex with the closed form of Syntaxin 1 has revealed that the Cdk5 phosphorylation site at Thr^{574} is inaccessible (Misura et al., 2000). Therefore, intermediate steps are likely to be present before Cdk5 phosphorylation, which might then regulate Munc18-1 binding to the assembling SNARE complex (Dulubova et al., 2007; Shen et al., 2007; Zilly et al., 2006). Munc13-1 is a strong candidate to initiate loosening of the Syntaxin 1 and Munc18-1 interaction and SNARE complex formation, as it is capable of binding both proteins and displace Munc18-1 from Syntaxin 1 (Betz et al., 1997; Gladycheva et al., 2004; Sassa et al., 1999). This may also involve RIM interactions with Munc13 (Betz et al., 2001). Munc18-interacting protein (Mint) appears to associate with Munc18-1 at some stage, possibly in Syntaxin-independent actions (Biederer & Südhof, 2000; Ciufo et al., 2005; Ho et al., 2006; Schutz et al., 2005). Furthermore, the vesicle-associated DOC2 translocates to the plasma membrane in a Ca^{2+}-dependent manner and binds to Munc18-1 and Munc13 (Groffen et al., 2006; Orita et al., 1997; Verhage et al., 1997). Interestingly, there is a Cdk5 phosphorylation consensus site in Munc13-1 (Barclay et al., 2004). Phosphorylation of Syntaxin 1 by Death Associated Protein kinase (DAP-kinase) also significantly reduces Munc18-1 binding (Tian et al., 2003). Thus, phosphorylation may in several ways facilitate initiation of SNARE complex formation (Deak et al., 2006; Fletcher et al., 1999). Munc18-1 has also been implicated as one of the proteins operating at the actual fusion event, but exactly in what way is still elusive and it might be unrelated to Syntaxin (Ciufo et al., 2005; Fisher et al., 2001). However, it has been demonstrated that both PKC and Cdk5 phosphorylation of Munc18 regulate the rate of expansion and opening time of the fusion pore in chromaffin cells (Barclay et al., 2004; Barclay et al., 2003). Phosphorylation narrows the kinetics of fusion pore opening, suggesting increased kiss-and-run exocytosis. The late role of Munc18-1 may be via regulation of Phospholipase D (PLD), an enzyme generating phosphatidic acid. PLD1 positively affects both phases of insulin secretion at a late step of exocytosis (Hughes et al., 2004). In neurons and chromaffin cells Munc18-1 has been shown to inhibit PLD prior to stimulation, after which inhibition is relieved (Lee et al., 2004a).

Docking of LDCVs seems highly Munc18-1-dependent, unlike in synapses where docking of SVs appears normal in the absence of Munc18-1 (Korteweg et al., 2005; Toonen et al., 2006a; Voets et al., 2001). This difference may in part be explained by the specific dependence of Syntaxin 1 for docking, as demonstrated in chromaffin cells (de Wit et al., 2006). It also involves actin rearrangements, but this seems independent of Syntaxin 1 (Nili et al., 2006; Toonen et al., 2006a). Granuphilin, which shows structure homology to Rabphilin3, is specifically expressed in beta cells and pituitary tissue and is involved in the tethering/docking step. Granuphilin is associated with granules and binds to the closed conformation of Syntaxin 1 and Munc18-1 and may in this way participate in Munc18-1-dependent docking (Coppola et al., 2002; Torii et al., 2004; Yi et al., 2002). Rab27a, which is associated with granules, interacts with Granuphilin but also with Myosin Va and via actin controls beta cell granule movement (Ivarsson et al., 2005; Izumi et al., 2003).

Hence, Munc18-1 promotes exocytosis also in endocrine cells. Similarly, in Munc18-1 deficient anterior pituitary cells hormone release was reduced (Korteweg et al., 2005). This was accompanied by a lining up of LDCVs away from the plasma membrane, suggesting defective docking in the absence of Munc18-1. We believe that Cdk5 phosphorylation of Munc18-1 occurs after initiation of SNARE complex assembly as detected by capacitance recordings. However, given the multitude of functions that the Munc18-1 protein appears to have, the Cdk5 phosphorylation might also be operating at several other events influencing insulin secretion. Information on possible biological differences between the Cdk5 activators p35 and p39 is limited but the proteins differ structurally in that p39 has a longer C-terminal sequence (Tang & Wang, 1996). That only p39 in complex with Cdk5 can promote Ca^{2+}-stimulated insulin exocytosis and mediate phosphorylation of Munc18-1 suggests p39 as an important activator in hormone release. Accordingly, in endocrine anterior pituitary cells p39 was found to be the predominantly expressed activator (Xin et al., 2004). Trio, a Rho GDP/GTP exchange factor (GEF) activating Rac, was demonstrated to be a Cdk5 substrate in these cells (Xin et al., 2004). Inhibition of Cdk5 hindered granule movement to the plasma membrane, a result presumably related to the role of Cdk5 in reorganizing actin cytoskeleton, which might have a similar function in insulin release. Thus, the Cdk5/p39 kinase appears to have a promoting role in hormone release that also needs to be investigated in neuronal cells. In addition, in lens epithelial cells p39 associates with Muskelin and Myosin II that are connected to actin, further implicating Cdk5/p39 in cytoskeletal rearrangements (Ledee et al., 2005; Ledee et al., 2007). At least the interaction with Muskelin occurs via the C-terminal part of p39 that is absent in p35, indicating a p39-specific role.

4.5 PRELIMINARY RESULTS

To further explore the roles of Munc18 proteins in beta cells we investigated the closest homolog of Munc18-1; Munc18-2. Munc18-2 shows high structure similarity and approximately 67% sequence identity to Munc18-1 (Hata & Südhof, 1995; Katagiri et al., 1995; Tellam et al., 1995). Munc18-2 has mostly been studied in epithelial tissues where it is the main Munc18 isoform but it is reportedly present in low amounts in neuronal, neuroendocrine and endocrine cells (Korteweg et al., 2005; Martin-Verdeaux et al., 2003; Riento et al., 1996; Steiner et al., 2002). We compared Munc18-1 and Munc18-2 mRNA levels and subcellular distribution of the proteins in beta cells. Furthermore, we investigated the influence of Cdk5 phosphorylation on subcellular localization. Due to the promiscuity of the available Munc18 antibodies for the two isoforms, we have created tagged constructs of intact and mutated templates to ensure that we were specifically studying Munc18-1 or Munc18-2.

4.5.1 Munc18 and Syntaxin isoform levels in brain and beta cells

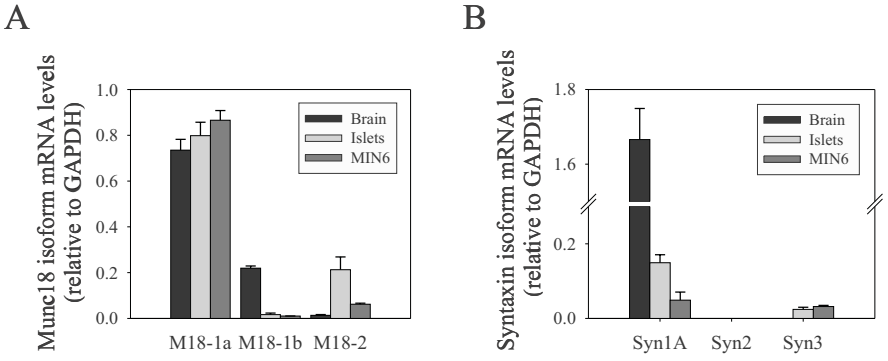

Figure 3. Semi-quantitative RT-PCR of mRNA transcripts of Munc18 **(A)** and Syntaxin **(B)** isoforms in *ob/ob* brain, *ob/ob* islets and MIN6 cells. Note different scaling in the graphs. Staples are representing the mean of RNA from three different animal or cell preparations (each repeated three times) and bars represent ± S.E.M.

RT-PCR amplification showed that the mRNAs of both splice variants of Munc18-1, Munc18-1a and Munc18-1b, but also Munc18-2 were transcribed in mouse *ob/ob* islets and the mouse insulinoma MIN6 cell line, as well as in *ob/ob* brain (not shown). Semi-quantitative RT-PCR using GAPDH as internal control showed that Munc18-1a mRNA was abundant and of similar levels in *ob/ob* brain, islets and MIN6 cells (Figure 3A). Levels of Munc18-1b were lower and barely detectable in beta cells. Munc18-2 is poorly expressed in brain but seems to be more abundant in pancreatic islets and MIN6 cells. Since Munc18 proteins function as accessory regulators via interactions with Syntaxins, we investigated the presence and abundance of the described Syntaxin partners of Munc18-1 and Munc18-2, Syntaxins 1-3, in pancreatic beta cells (Katagiri et al., 1995; Pevsner et al., 1994; Riento et al., 1998). RT-PCR with Syntaxin isoform specific primers detected Syntaxin 1A, 2 and 3 (not shown). With semi-quantitative RT-PCR Syntaxin 1A expression was found to be prominent

in brain, quite low in islets and yet lower in MIN6 cells, comparable to Syntaxin 3 levels in islets and MIN6 cells (Figure 3B). Syntaxin 3 mRNA in brain and Syntaxin 2 in all tissues analyzed were below the detection level in the assay. Syntaxin 2 has been reported to be poorly expressed in beta cells and was not coupled to insulin exocytosis (Nagamatsu et al., 1997). Although Munc18-1 and Munc18-2 can bind Syntaxin 1-3 the affinities for all interactions may not be equal. It is tempting to propose Syntaxin 3 as a partner of Munc18-2 in beta cells, as has been shown for epithelial and mast cells (Fukuda et al., 2005; Martin-Verdeaux et al., 2003; Riento et al., 2000). In the insulinoma beta cell line HIT Syntaxin 3 has also been detected as a cytoplasmic protein in contrast to other plasma membrane Syntaxins (Kang et al., 2002).

4.5.2 Subcellular localization of Munc18 protein variants in MIN6 cells

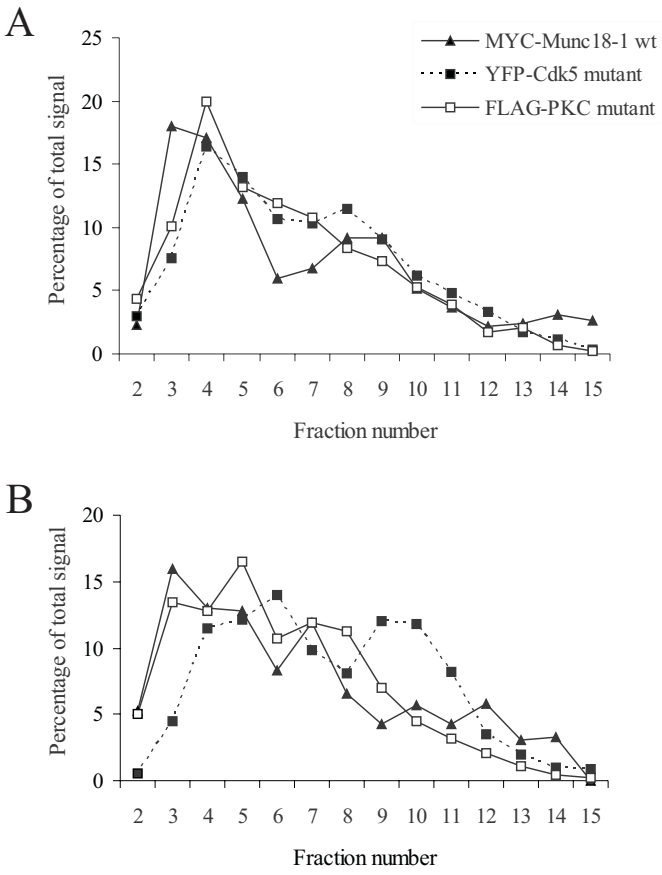

Figure 4. Subcellular fractionation distribution of Munc18-1 wt and Munc18-1 mutated in PKC and Cdk5 phosphorylation sites in unstimulated (0.5 mM glucose) **(A)** and glucose-stimulated (25 mM glucose) **(B)** MIN6 cells. Na^+/K^+ ATPase α-1 is used as plasma membrane marker and is typically associated with fractions 4-8. All gradients were repeated three times and Western blotting performed twice per condition.

By stimulating MIN6 cells with glucose and performing sucrose density gradient analysis, we investigated the localization and mobility of wt and mutated Munc18 proteins under resting conditions and after increased exocytotic activity. To ensure that co-transfection with Syntaxin 1 was not needed for proper localization of overexpressed Munc18-1 or Munc18-2 we also performed immunocytochemistry of either Munc18 single transfections or in combination with Syntaxin 1 (data not shown). The distribution of Munc18-1 wt in unstimulated MIN6 cells is broad (Figure 4), with no apparent membrane accumulation (37%). However, after glucose-stimulation there was a translocation of Munc18-1 wt to the plasma membrane (66%, $p< 0.01$). Interestingly, in unstimulated cells the plasma membrane localization of the Munc18-1 Cdk5 and PKC phosphorylation mutants (63% and 55%) was higher than for wt ($p< 0.05$), comparable instead to wt levels in stimulated cells. Degree of plasma membrane localization for the mutated constructs was not altered after stimulation (66% and 62%). Thus, Munc18-1 is not stably associated with membranes but recruited and translocated after stimulation and phosphorylation/dephosphorylation may be needed for Munc18-1 cycling at the plasma membrane also in resting cells. In yeast the Munc18 homolog Vps45p dissociates from the membrane in the absence of PP1 activity but reassociates after its reactivation (Bryant & James, 2003). Both PP1 and PP2B remove PKC-mediated phosphorylation of Munc18-1 (Craig et al., 2003), but which phosphatase that regulates Cdk5 action is not known. Phosphorylation of Munc18-1 appears to be required in beta cells under basal conditions, as the mutated proteins are differently located than Munc18-1 wt. As demonstrated in PC12 cells, less Munc18-1 protein is bound to Syntaxin than the high affinity suggests and maybe phosphorylation is involved in regulating its localization (Zilly et al., 2006). In MIN6 cells apparently exocytotic activity is needed for recruitment of Munc18-1 to the plasma membrane, as glucose-stimulated cells show a significant increase in membrane located Munc18-1. PKC phosphorylation of Munc18-1 was shown to be low under basal conditions in brain terminals but to be induced by Ca^{2+}, with no detected prominent change in Cdk5 phosphorylation (de Vries et al., 2000). Interestingly, PKC phosphorylation, as well as increased levels of Munc18-1, were shown to stimulate replenishment of the RRP in an activity-dependent manner but were not necessary for release, nor docking (Nili et al., 2006; Toonen et al., 2006b). What regulates Cdk5 activity besides the activator proteins is not well recognized. However, in neuroendocrine cells depolarization caused translocation of Cdk5 from the cytosolic to a particulate cellular compartment (Fletcher et al., 1999). Similarly, in a previous study we detected translocation of Cdk5 after glucose-stimulation in beta cells (Lilja et al., 2001). Further comparisons of Cdk5 and PKC phosphorylation of Munc18-1 between neurons and beta cells would be valuable to delineate potential differences in functions for these cell types.

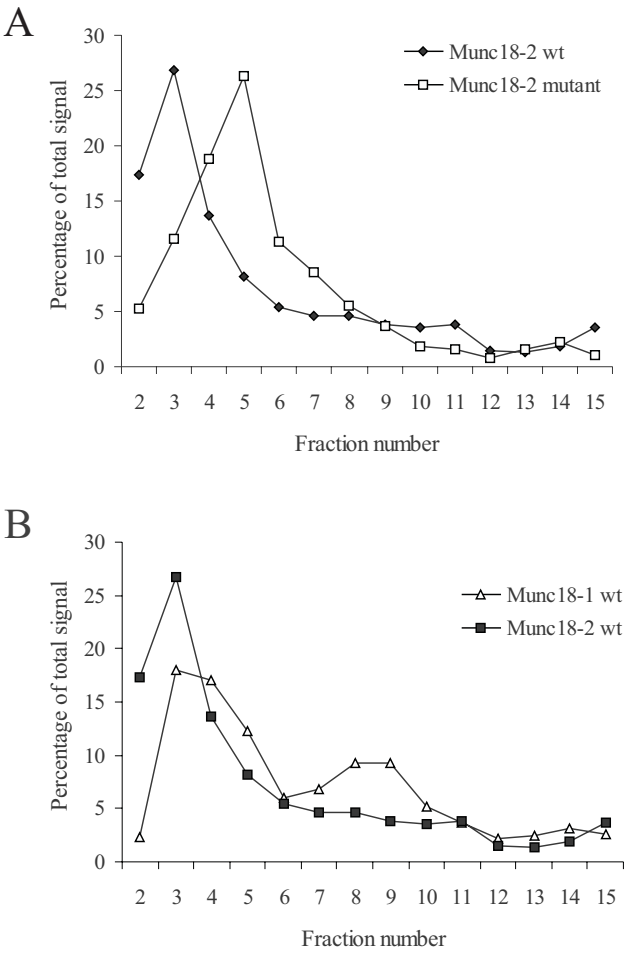

Figure 5. A, sucrose gradient analysis of Munc18-2 wt and Munc18-2 Cdk5 phosphorylation mutant distributions in unstimulated MIN6 cells. No obvious differences were detected under stimulatory conditions (data not shown). **B**, comparison of subcellular distribution of Munc18-1 and Munc18-2 wt proteins in unstimulated MIN6 cells.

We identified a putative Cdk5 phosphorylation site in Munc18-2, located at a corresponding position as the equivalent in Munc18-1. To explore if Munc18-2 is a Cdk5 substrate we created a mutated construct potentially unable to become phosphorylated (Thr $^{573}\rightarrow$ Ala^{573}). As for Munc18-1, we investigated the subcellular localizations of transfected Munc18-2 wt and the Cdk5 phosphorylation mutant in MIN6 cells (Figure 5A). Equally low amounts of both wt and mutated Munc18-2 were present in plasma membrane fractions (~24%) in unstimulated cells. There was no apparent translocation to the plasma membrane after glucose-stimulation for either wt or mutated Munc18-2 proteins (~26%). Munc18-2 was located almost exclusively in low-density fractions. However, the Cdk5 phosphorylation mutant peaked in a higher-density fraction (fraction 5) than wt protein (fraction 3). This suggests that Cdk5 is phosphorylating Munc18-2, and similar to Munc18-1, that it has a role in trafficking and cycling of Munc18-2. Comparing Munc18-1 and Munc18-2 wt protein subcellular localizations showed that Munc18-2 was localized to lower-density fractions than

Munc18-1 (Figure 5B) and less associated with plasma membrane fractions ($p<0.01$). A thorough examination of co-localization of the different Munc18 variants with subcellular markers will be required to determine their precise cellular localization. Given the differences in localization pattern we speculate that Munc18-2 has a different role than Munc18-1 in beta cells. The higher expression levels of Munc18-2 in beta cells suggest a specific function in vesicle trafficking not present in neurons or neuroendocrine cells. Prominent Munc18-2 expression was also recently detected in endocrine anterior pituitary cells (Korteweg et al., 2005). This function is presumably not associated with fusion events at the plasma membrane. In mast cells Munc18-2 is connected to microtubules and stimulation of beta cells also induces granule movement dependent on microtubule cytoskeleton in addition to actin (Martin-Verdeaux et al., 2003; Rutter & Hill, 2006). In amylase secreting acinar cells the tethering/docking factor Granuphilin interacts with Syntaxin 2/3 in a Munc18-2-dependent manner (Fukuda et al., 2005). Thus, Munc18-2 may function in mobilization of granules from reserve pools. Alternatively, it may be involved in the second phase of biphasic insulin exocytosis by recruitment of granules for fusion by an alternative route where the docking requirement is bypassed (Ohara-Imaizumi & Nagamatsu, 2006). Our results further suggest multiple involvement of Cdk5 phosphorylation of SM proteins in insulin secretion, not only as a molecular switch to control Syntaxin 1. How vesicles are recruited and selected to the fusion sites is not well characterized in beta cells but we have now identified a new potential mediator of trafficking in insulin exocytosis. Whether Munc18-2 function in beta cells involves a Syntaxin partner and how it is regulated by phosphorylation needs to be determined.

5 CONCLUSIONS AND GENERAL PERSPECTIVE

This thesis aimed at exploring differences, at the molecular level, in exocytotic events. Specifically we were interested in investigating various types of exocytosis; neurotransmitter release and hormone secretion.

The first part focused on the SNAP-25 protein, an essential part of the fusogenic SNARE complex, and the functional difference between the two isoforms SNAP-25a and SNAP-25b. This was assayed in a developed mouse mutant where SNAP-25b, the major isoform in adult brain and important for evoked neurotransmission, was replaced with supplementary SNAP-25a, a developmental earlier isoform that is also functioning in neuroendocrine and endocrine exocytosis. The two resultant SNAP-25b mouse mutants express only SNAP-25a at different but close to physiological levels of total wt SNAP-25. We discovered that reduced levels of SNAP-25a in unfloxed mice during early postnatal development, results in a severe phenotype with reduced growth, hyperactivity, frequent seizures and early lethality. In floxed mice with restored total levels of SNAP-25 no evident developmental defects were observed. However, both models demonstrated reduced short-term plasticity in the hippocampus, which was overcome at higher stimulation frequencies in floxed mutants. With time, seizure activity developed also in floxed mice and there were long-term alterations in neuropeptide and BDNF expression in the hippocampus. Adult floxed mice show cognitive impairments with increased anxiety and deficits in spatial learning.

Our results demonstrate that *in vivo*, the alternative switch in splicing from SNAP-25a to SNAP-25b is essential for developing and maintaining accurate synaptic performance. We speculate that impairments manifested in our SNAP-25b null mutants are consequences of early events during synapse formation and development of neuronal circuitry. The SNAP-25a protein is apparently incapable in supporting both normal short- and long-term synaptic plasticity which is affecting cognitive functions. The disturbed synaptic function launches seizure activity that initiates neuroprotective mechanisms with altered expression of neuropeptides and neurotrophic factors. Gradually, abberrant neuronal activity becomes manifested as evident morphological changes in susceptible and highly plastic brain areas. Our unfloxed mouse model provides a tool for investigating vurnerability to levels of SNAP-25 and aberrant exocytosis during development. Functional differences in signaling and release properties for SNAP-25a and SNAP-25b containing SNARE complexes can be addressed in neuronal cells using the floxed mouse model. In future studies, protein interactions with SNAP-25a and SNAP-25b at the molecular and biochemical level can be evaluated under *in vivo*-like conditions. Furthermore, the floxed animals can be useful for investigating the importance of accurate neuronal signaling and plasticity for degenerative processes.

The second part of the thesis concerned Cdk5, a kinase that was initially identified as a kinase important for neurite outgrowth but is emerging as a regulator of cell signaling. To study Cdk5 in neuronal exocytosis, we investigated Cdk5 phosphorylation and the role of the activators after initial establishment of synaptic contact in an *in vitro* model of the neuromuscular junction. In the NG108-15 cell line Cdk5 and p35 protein levels were prominent after cellular differentiation and both proteins were preferentially distributed to soluble pools. p39 was expressed at low levels and primarily present in membrane and cytoskeletal fractions. A dominant

negative Cdk5 inhibited spontaneous transmitter release. Overexpression of either Cdk5 activator p35 or p39 increased both the number of functional synapses and the frequency of spontaneous neurotransmitter release, though p39 was more potent.

Diabetes mellitus is a disease affecting millions of people worldwide and in Type II diabetes insulin release is impaired. We have previously identified Cdk5 as a component and enhancer of pancreatic beta cell exocytosis (Lilja et al., 2001) and now further explored this action of Cdk5. We found that both Cdk5 activators are expressed in primary mouse beta cells and that they are distributed to membranous organelles of partly different densities. Munc18-1 is a Cdk5 substrate essential for neuronal exocytosis that we identified as abundant in beta cells. The activator p39 potentiates Cdk5 function in Ca^{2+}-dependent exocytosis in beta cells via phosphorylation of Munc18-1. We demonstrated that in mouse pancreatic beta cells, in contrast to in neuronal and neuroendocrine cells, expression of the Munc18-2 isoform is prominent in addition to Munc18-1. Munc18-2 exhibits a different subcellular localization than Munc18-1 and selectively Munc18-1 was translocated to the plasma membrane after glucose-stimulation. This suggests the recruitment of Munc18-1 under exocytotic activity and a separate and specific function for Munc18-2 in granule trafficking. Phosphorylation mutants of Munc18-1 and Munc18-2 showed different subcellular localization compared to wt proteins, implicating the importance of phosphorylation in Munc18 cycling and that Munc18-2 is a Cdk5 substrate.

Taken together, Cdk5 promotes exocytosis in both neuronal and pancreatic beta cells. Activity might be regulated by the expression levels and localization of the activator proteins and specifically p39 potentiates beta cell exocytosis and phosphorylation of Munc18-1. We speculate that also in neuronal cells Munc18-1 is a substrate of Cdk5/p39. Few substrates of p39 have so far been identified and this activator could be prominent in hormone and peptidergic release. Munc18-2 might have a specific role in trafficking in endocrine cells that may involve Cdk5 phosphorylation.

Exocytotic protein isoforms are increasingly being recognized as providing cells with differential release properties. The precise temporal and spatial regulation of expression is important for neuronal diversity and plasticity. Hormone release is based on the same molecular components as neurotransmitter release but can be equipped with certain protein variants. Furthermore, modification of exocytotic proteins by posttranslational events such as phosphorylation provides additional and immediate regulation that may translate into diversity in release properties. It is becoming clear that disturbances in release underlie a number of neuropsychiatric disorders and hormonal imbalances in the body and understanding of exocytosis is vital for improving the treatment of such diseases.

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