General Discussion

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The aim of this thesis was to understand how a neuron controls synaptic strength. We focused on the role of two important proteins in this process, liprin-α2 and Munc18-1. We chose these two proteins because the liprin family functions as a master controller of active zone development in lower organisms and Munc18-1 is one of few proteins essential for synaptic vesicle release. Of particular interest were the regulation of liprin-α2 and Munc18-1 by posttranslational modifications such as phosphorylation and ubiquitination.

First, we identified liprin-α2 as an important and upstream modulator of active zone composition in mature neurons (*Chapter 2*). We found that liprin-α2 levels determine synaptic strength by regulation of RRP size. We show that liprin-α2 turnover in synapses strongly depends on proteasomal degradation via the APC/C complex.

Next, we showed the importance of ERK in the regulation of the presynaptic protein Munc18-1. We identified a pathway in which high neuronal activity leads to presynaptic ERK activation via retrograde endocannabinoid signaling. ERK in turn phosphorylates Munc18-1, which triggers the recruitment of the E3 ligase subunit Fbxo41. Subsequent proteasomal degradation of Munc18 decreases synaptic strength. Hence, Munc18 functions in negative feedback regulation to contribute to homeostasis in the brain (*Chapter 3*).

Chapter 4 dealt with the role of Cdk5 phosphorylation of Munc18-1 in neurons. We show that Cdk5 phosphorylation of Munc18-1 plays an important role in the regulation of synaptic strength, similar to what has been found in secretory cells (Fletcher et al., 1999; Lilja et al., 2001; Shuang et al., 1998). Hence, Cdk5 phosphorylation of Munc18-1 does not underlie Cdk5-dependent silencing of synapses, but may play a role in neuronal silencing induced increase of presynaptic strength.

Finally, we established a tool for automated analysis of neuronal morphology, which has been used in all other chapters. The software routine analyzes morphological and protein expression data for soma, neurites and synapses from immunofluorescent images in a time-efficient manner (*Chapter 5*).

Homeostatic modulation of presynaptic strength – the role of liprin-α2 and Munc18-1

Neurons modulate synaptic strength in response to changes in neuronal activity. Generally, high neuronal activity decreases synaptic strength, whereas neuronal silencing increases synaptic strength. These homeostatic changes can have both pre- and postsynaptic origin, as shown by changes in mEPSC frequency and amplitude, respectively (Pozo and Goda, 2010; Turrigiano, 2011). Postsynaptic regulation of receptor numbers and postsynaptic density composition upon activity changes has been studied extensively (Ehlers, 2003; Wierenga et al., 2005). In contrast, the importance of modulation of active zone protein levels at the presynapse has only recently been explored (Figure 6.1). Postsynaptic silencing results in upregulation of several presynaptic proteins (RIM, SV2B, Syt1, Cav2.1, vGluT) whereas others are downregulated (Bassoon, piccolo, ELKS/CAST, Munc13, synapsin and liprin-α) (De Gois et al., 2005; Lazarevic et al., 2011). Thus, activity changes lead to similar differential regulation of subsets of proteins at the pre- and postsynaptic site (Ehlers, 2003).

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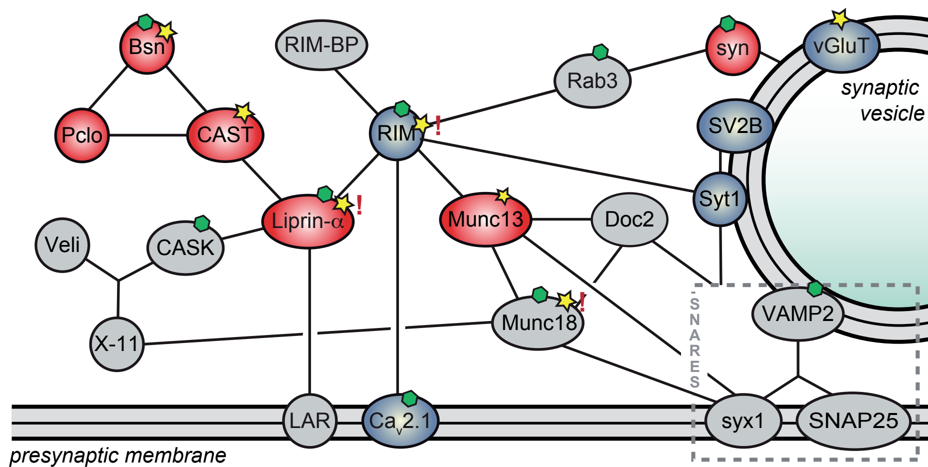
What is the mechanism that controls these changes in synaptic protein levels?

Increased synaptic levels of the priming factor RIM in silenced neuronal cultures are not accompanied by similar changes in total RIM levels (Lazarevic et al., 2011). Therefore, synaptic recruitment of RIM to (a subpopulation of) synapses rather than protein translation likely underlies upregulation of synaptic RIM levels. Increased levels of synaptic-vesicle proteins Syt1, vGluT and SV2B are likely due to recruitment of synaptic vesicles and increased pool size upon neuronal silencing. In addition to these recruitment mechanisms, recent work has established a role of the ubiquitin-proteasome system (UPS) in the regulation of active zone composition and synaptic strength during homeostatic plasticity (Ehlers, 2003; Jiang et al., 2010; Rinetti and Schweizer, 2010; Willeumier et al., 2006). Inhibition of the proteasome for six hours results in increased synaptic levels of Bassoon, ELKS/CAST, Munc13, RIM, synapsin and liprin-α indicating that these proteins and most likely many more in the presynapse are regulated by active proteolytic breakdown via ubiquitination



Regulation of active zone composition

higher synaptic levels upon neuronal silencing

lower synaptic levels upon neuronal silencing

not modulated/not studied

levels changed after proteasome block

! E3 ligase identified

levels changed in Lirpin-α2 KD neurons

Figure 6.1: Regulation of active zone composition. The graph depicts proteins (circles) and their interactions (lines) at the presynaptic active zone. Upon postsynaptic silencing, synaptic protein levels are differentially regulated and are either increased (blue) or decreased (red). Synaptic levels of several proteins are changed upon proteasome block (yellow star), but only few E3 ligases that mediate the regulation have been identified to date (exclamation mark). Green hexagons indicate proteins that showed decreased synaptic levels in liprin-α2 knockdown neurons (Chapter 2).

processes (Lazarevic et al., 2011). In this process substrate recognizing E3 ligases play an essential role. Currently, only the E3 ligases for RIM and liprin-α, SCRAPPER and APC/C, respectively, have been identified (van Roessel et al., 2004; Yao et al., 2007). In this thesis we identified Fbxo41 as the E3 ligase that controls Munc18-1 levels (Chapter 3). It remains unknown whether other proteins, whose synaptic levels are changed upon proteasome block, are also ubiquitinated and degraded directly. As an alternative and perhaps more economical mechanism, essential proteins such as Munc18-1, liprin-α or RIM that interact with several other active zone proteins may serve as central protein 'hubs'. Degradation of few 'hubs' could be a quick and economical way to regulate active zone composition and synaptic strength.

Chapter 2 shows that liprin-α2 knockdown dramatically decreases synaptic strength. Loss of liprin-α2 reduces synaptic levels of several direct and indirect liprin-α2 interactors in the active zone, showing that liprin-α2 functions upstream of these proteins. Furthermore, liprin-α2 protein levels are regulated during homeostatic plasticity paradigms and synaptic turnover via APC/C-dependent proteasomal degradation. Together, we show that liprin-α2 functions as protein hub upstream of other active zone proteins and that it modulates integrity and localization of protein complexes in the synapse.

Homeostatic modulation of presynaptic strength – Outstanding questions

Synapses operate as independent units and can individually tune their strength. To understand this local homeostatic plasticity, it is essential to observe protein recruitment, translocation, degradation and translation at the level of single synapses. Knock-in (KI) mice, expressing fluorescently labeled versions of presynaptic proteins from the endogenous gene locus, are powerful tools to study the molecular dynamics at the single synapse level. Fluorescence-recovery after photo-bleaching (FRAP) in these synapses will give insight in the molecular dynamics of proteins in a minimally perturbed system. Pharmacological perturbation of neuronal activity, but even better single synapse stimulation using photo-manipulation or compound uncaging, will further identify mechanisms by which synapses regulate presynaptic protein composition and hence synaptic strength. Currently, Munc18-Venus KI mice are analyzed in the lab. In addition, liprin-α2 and RIM1 knock-in mice would be valuable assets to investigate the role of these protein hubs in more detail. In the near future, *in vivo* optical analysis of molecular dynamics in freely moving animals will be a challenging though extremely powerful way to understand the mechanisms by which neurons fine-tune their synapses. The ultimate goal would be to finally link these molecular events to the behavior of the whole organism.

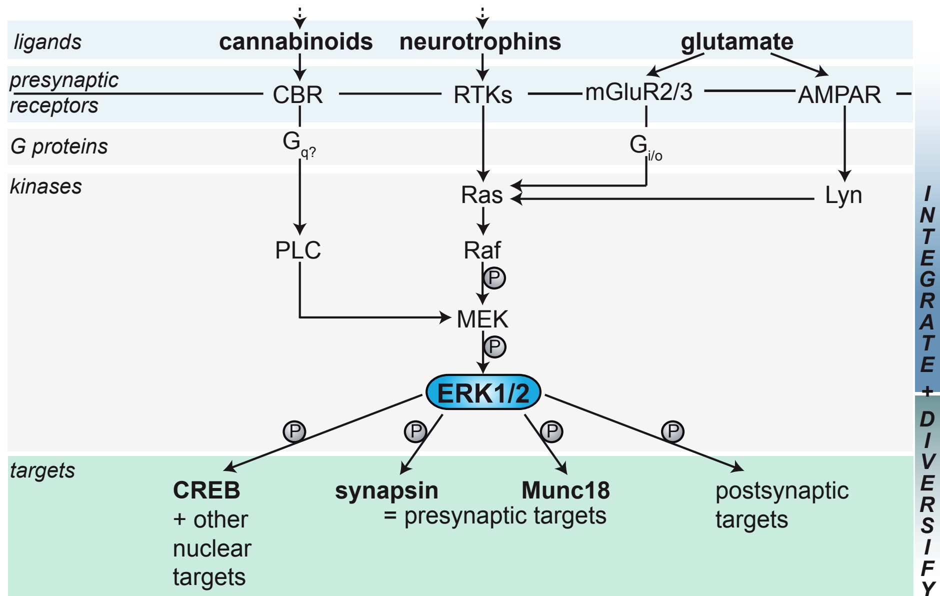


Figure 6.2: ERK as central component between signal integration and diversfication.
Activation of several receptors leads to activation of ERK via a sequence of phosphorylation events (*integration*). ERK activation triggers the phopshorylation of nuclear, presynaptic and postsynaptic targets (*diversification*).

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Many receptors, many targets – ERK provides synapse specificity

Posttranslational modification pathways are often activated in different ways and may thus provide the necessary synapse specificity described above. ERK is activated upon neurotrophin, endocannabinoid as well as glutamate receptor activation (Derkinderen et al., 2003; Ferraguti et al., 1999; Gómez and Cohen, 1991; Sugden and Clerk, 1997). Similar to release probability (Branco and Staras, 2009), receptor subtype expression varies widely between synapses (Luján et al., 1997; Macek et al., 1996; Nusser et al., 1998). Synapse-specific expression of receptor subset and number therefore allows fine-tuning and synapse-specific modulation of ERK activation. In addition, even different concentrations of the same receptor agonist (as seen for BDNF (Ji et al., 2010)) result in different activation patterns of ERK. Amplitude and duration of ERK activation in turn determine its biological outcomes (Ji et al., 2010; Wu

et al., 2001). Hence, synaptic ERK activation depends on spatial and temporal characteristics of the upstream signaling components and implies a function as coincidence detector at the synaptic level.
Furthermore, ERK phosphorylates a variety of postsynaptic, presynaptic and nuclear targets (Grewal et al., 1999). Target phosphorylation therefore depends on subcellular localization, translocation and activity of both target proteins and kinase. In addition, the presence of specific phosphatases that counteract phosphorylation will also contribute to the spatio-temporal regulation of ERK phosphorylation.
Together, ERK is a central component between integration and diversification of signals (Figure 6.2) and therefore plays an important role in functional diversity and temporal regulation of individual synapses.

The role of Munc18-1 in synaptic transmission

Chapters 3 and *4* describe in detail the effects of post-translational modifications of Munc18-1 on synaptic transmission. In the following paragraphs I will try to put these mechanistic insights into a bigger perspective.

Is Munc18-1 involved in fear extinction?
Chapter 3 describes an activity-dependent pathway that leads to the production of endocannabinoids and ERK-dependent phosphorylation of Munc18-1. Endocannabinoids are known to function as retrograde messengers since they are produced in the postsynaptic cell, diffuse through membranes and activate CB1 receptors on the presynaptic terminal (Wilson et al., 2001; Wilson and Nicoll, 2001, 2002). We show that Munc18-1 phosphorylation by ERK, which is activated upon CB1R activation, functions in the initial phase of cannabinoid induced synaptic depression. Such negative feedback loops, commonly known as homeostatic plasticity, are important to keep network activity stable over a long period of time and to prevent overexcitability of the brain (Turrigiano, 2011) (Figure 6.3). An important next step will be to understand the *in vivo* role of this pathway. To start, contextual fear conditioning and fear extinction paradigms could be used to assess the role of this pathway in fear behavior since several components of this pathway play a role in in fear behavior:

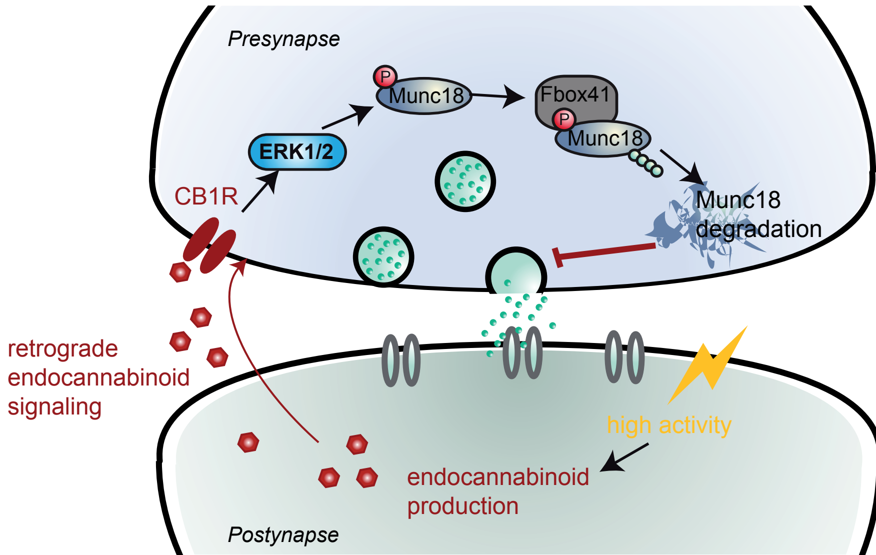

Figure 6.3 Munc18-1 in retrograde endocannabinoid signaling.
Upon high activity, postsynaptic endocannabinoids bind to presynaptic CB1 receptors and trigger a cascade that leads to ERK activation, Munc18 phosphorylation and degradation that decreases neurotransmitter release.

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The endocannabinoid system has been implicated in anxiety, stress responses and fear learning and is generally believed to protect an organism from over-reaction to aversive events (for review see: Riebe and Wotjak, 2011; Ruehle et al., 2011). Several studies using CB1R null mutant animals or injections of the CB1R agonist WIN55,212-2 into the basal lateral amygdala or CA1 region of the hippocampus before exposing rats to a stressor show that activation of the endocannabinoid system is important in the regulation of fear extinction (Abush and Akirav, 2010; Ganon-Elazar and Akirav, 2009, 2011; Marsicano et al., 2002; Varvel and Lichtman, 2002).
In addition, the ERK pathway is activated during fear extinction or prolonged stress (Fischer et al., 2007; Xiao et al., 2011) and this activation is dependent on CB1R signaling (Cannich et al., 2004). Injection of the ERK inhibitors PD98059 or U0126 into the hippocampus before fear extinction shows that ERK activity is important for the extinction of fear. Animals in which ERK has been inhibited keep responding to the fear stimulus and thus do not show fear extinction (Fischer et al., 2007).
Since both endocannabinoids and ERK function in the Munc18-dependent

homeostatic plasticity pathway described in *Chapter 3*, phosphorylation of Munc18-1 may play an important role in the extinction of fear. This hypothesis could be tested with mice that instead of wild-type Munc18-1 express the non-phosphorylatable mutant $M18_{S241A}$ in the hippocampus or amygdala. To this end, adenoviruses expressing Munc18 variants together with Cre recombinase should be injected into these brain areas of conditional Munc18-1 knockout mice. These mice are currently available in the institute. Alternatively, small peptides that interact only with phosphorylated Munc18-1 could be injected to interfere with the pathway described in *Chapter 3*. Injections at different stages of behavioral testing will allow temporal control to distinguish involvement of Munc18-1 phosphorylation in different phases of fear behavior.

Is Munc18-1 involved in post-traumatic stress disorder?

In humans, extreme or life-threatening situations lead to acute but most often transient stress responses. However, some individuals suffer from long-term and severe functional impairments, generally described as post-traumatic stress disorder (PTSD). Initially PTSD was thought to be at the extreme end of a normal spectrum of responses to trauma correlating with the intensity of the stressor. However, it has become clear that in addition to the stressor, also factors specific to the individual contribute substantially to the development of PTSD (for review see: Sherin and Nemeroff, 2011). Twin studies provide evidence for a genetic predisposition (30-70%) for the development of PTSD after exposure to potentially traumatic events (Koenen et al., 2009; Sartor et al., 2011; Stein et al., 2002; True et al., 1993). Until now, only few genetic risk factors, associated with either dysregulation of the hypothalamic-pituitary-thyroid axis or abnormal release and/or regulation of different neurotransmitters, have been identified (Koenen et al., 2009). Nonetheless, knowledge of the molecular mechanisms underlying PTSD is largely missing (Sherin and Nemeroff, 2011). As with many complex disorders or traits, identification of single genes by genome-wide association study underlying PTSD pathology is difficult since many different genes with small effects might contribute to risk. Collective testing of genes involved in biological pathways has emerged as an alternative strategy for testing the combined effects of genetic variants with small effect size (Holmans et al., 2009; Ruano et al., 2010; Torkamani et al., 2008).

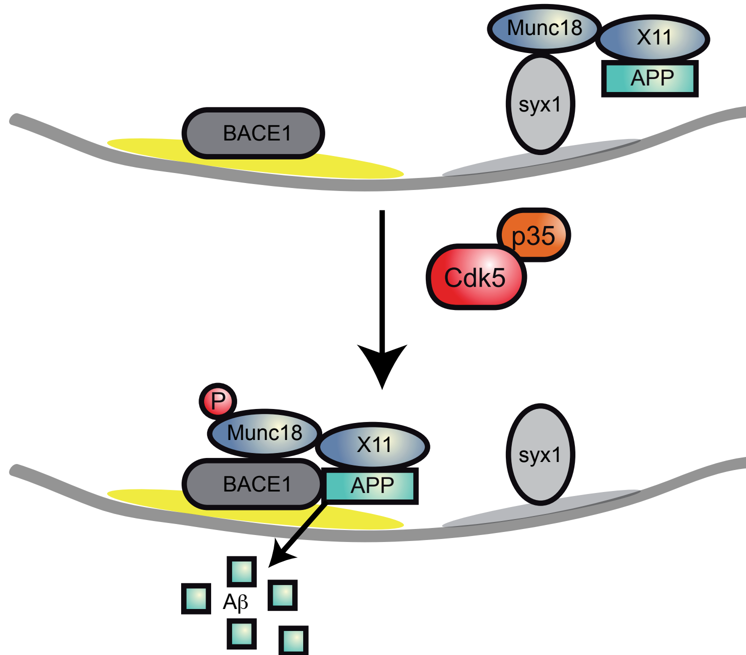

Figure 6.4 Model of the role of Cdk5 phosphorylation in APP cleavage. Normally, BACE and APP are localized in two different microdomains at the plasma membrane. Here, APP interacts with the syntaxin/Munc18/X11 complex. In Alzheimer's disease, hyperactivation of Cdk5 leads to increased phosphorylation of Munc18, which dissociates syntaxin/Munc18 binding. APP translocates to the BACE microdomain increasing Aβ42 production.

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The involvement of both ERK and endocannabinoids in extinction of fear together with the fact that the endocannabinoid pathway is highly regulated by stress hormones such as glucocorticoids (de Oliveira Alvares et al., 2010; Ursano et al., 2010) implies that dysregulation of the identified pathway in *Chapter 3* might increase the risk for PTSD. Furthermore, studies using plant-derived cannabinoids (e.g. THC from *Cannabis sativa*) report initial beneficial effects on PTSD symptoms (Fraser, 2009). Behavioral studies should investigate whether other proteins in this pathway might serve as drug targets and offer new therapeutic opportunities for PTSD patients.

Is Munc18-1 involved in Alzheimer's disease?

Chapter 4 describes the effect of Cdk5 phosphorylation of Munc18-1 on synaptic transmission. Excessive and prolonged presence of the Cdk5 activator p25 leads to hyperactivation of Cdk5, which has been linked to Alzheimer's disease (AD) pathology (Cruz and Tsai, 2004; Patrick et al., 1998; Patrick et al., 1999). AD is a progressive neurodegenerative disease that starts with mild synaptic dysfunction and memory problems and worsens in the time course of years to complete loss of cognition and executive function (for review see: Pimplikar et al., 2010).

Intracellular formation of neurofibrillary tangles and extracellular deposition of amyloid β (Aβ) plaques are the two major hallmarks of AD. Recent work suggests that Cdk5 might be involved in both processes: Hyperactive Cdk5 heavily phosphorylates the microtubule-associated protein tau, which is not a Cdk5 substrate under physiological conditions (Ko et al., 2001; Van den Haute et al., 2001). Hyperphosphorylated tau aggregates which leads to the formation of neurofibrillary tangles inside nerve cells (Abraha et al., 2000; Ding et al., 2006; Haase et al., 2004). In addition, Cdk5 hyperactivity enhances β-cleavage of APP via transcriptional regulation of BACE and thereby increases the amount of Aβ42, which in turn results in synapse dysfunction and memory loss via an unidentified mechanism. Cdk5 inhibition in transgenic p25 mice reduces Aβ42 production suggesting that Aβ42 processing is regulated by the Cdk5/p25 complex (Wen et al., 2008).

Importantly, Sakurai et al. (2008) show that Cdk5 phosphorylation of Munc18-1 may play a crucial role in this amyloid processing. Biochemical assays showed that localization of APP depends on Cdk5 phosphorylation of Munc18-1. Normally, APP is localized in syntaxin-containing microdomains together with Munc18-1 and Mint/X11. This prevents interaction of APP with β-secretase (BACE1) and thus the production of Aβ42. During hyperactivation of Cdk5, Cdk5 phosphorylation of Munc18-1 dissociates the syntaxin-Munc18-X11 complex and APP translocates to microdomains containing BACE1 (Sakurai et al., 2008). This translocation may lead to higher levels of Aβ42 in Alzheimer patients with hyperactive Cdk5 via a Munc18-dependent mechanism (Figure 6.2). In line with this hypothesis, in brain extracts from Alzheimer's patients, many proteins of this cascade are upregulated (Cdk5, Munc18-1, X11) compared to healthy controls (Jacobs et al., 2006). Thus, Munc18-1 phosphorylation

may provide an additional or alternative explanation for reduced synaptic output leading to synapse dysfunction. To obtain mechanistic insight into Munc18-1's role in Alzheimer's disease, the following questions should be addressed:

1) Does Munc18-1 interact directly with APP?
It is currently unclear whether the assembly of the syntaxin-Munc18-APP complex is dependent on the presence of X11 or whether Munc18-1 can directly bind APP. Yeast-two hybrid screens, immunoprecipitations and mass spectrometry analysis can be used to answer this question.

2) Does Munc18-1 play a role in APP trafficking?
The current hypothesis suggests that APP translocates to BACE1 after disruption of the syntaxin-Munc18-X11-APP complex. It remains elusive whether the complex dissociates entirely or whether Munc18-X11-APP stay together and traffic to BACE1. Imaging studies, including Förster Resonance Energy Transfer (FRET), will help to answer these questions and to study the dynamics of these complexes. Importantly, if Munc18 is needed to bring APP to BACE1, Aβ42 levels should be much lower in Munc18 KO mice compared to control.

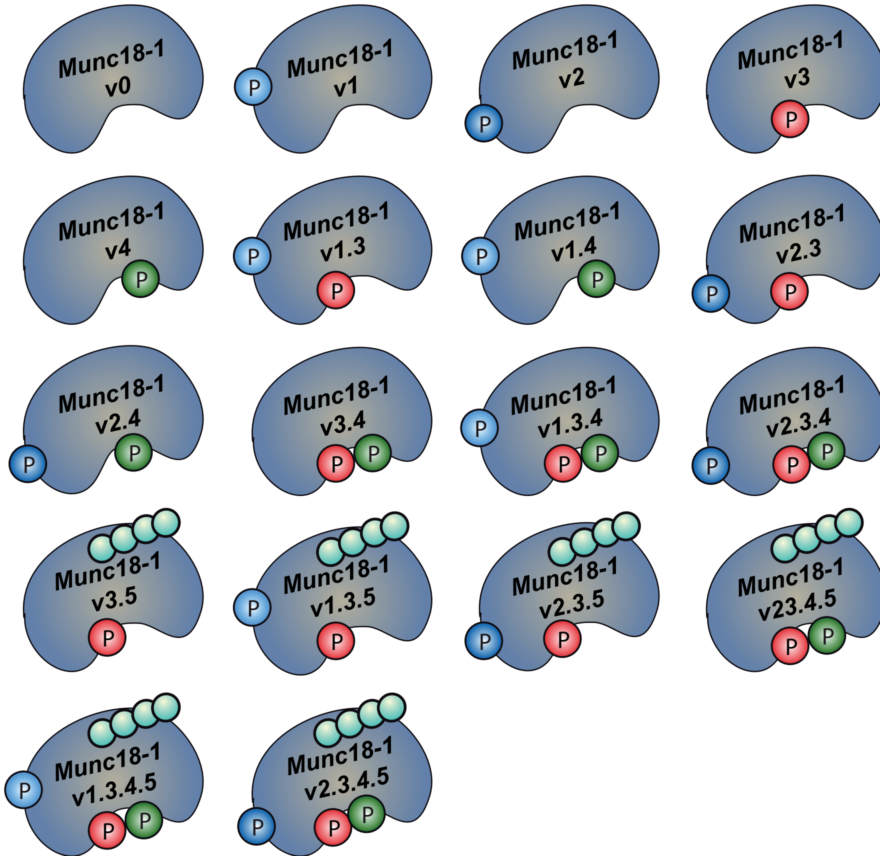
3) Does Munc18-1 phosphorylation by Cdk5 contribute to Alzheimer pathology independent of APP processing?
Chapter 4 showed that Cdk5 phosphorylation of Munc18 results in increased neurotransmitter release. During hyperactivation of Cdk5, excessive Munc18 phosphorylation might result in excitotoxicity. It is presently not understood whether the increased Cdk5 phosphorylation Munc18, contributes to the AD onset and/or disease progression in APP-dependent and/or –independent ways.

Involvement of Munc18 in epilepsy
Several recent studies have shown that Munc18-1 is linked to Ohtahara or West syndromes, two particular forms of epilepsy in young children (Deprez et al., 2010; Hamdan et al., 2011; Hamdan et al., 2009; Mignot et al., 2011; Milh et al., 2011; Otsuka et al., 2010; Saitsu et al., 2008). In these patients, single amino-acid changes have been identified in different domains of Munc18-1 (Figure 1.5). Currently it is not known

whether the epilepsy causing mutations affect neuronal connectivity or modulate neurotransmitter release directly or indirectly: Mutations could influence synapse number or neurite outgrowth or directly control synaptic transmission by changing the affinity for its binding partners. As another option, mutations could hinder modulation of Munc18 function by posttranslational modifications and render it unresponsive to changes in network activity. A recent study describes a patient in which the mutation in STXBP1 gene (1720A>C) results in an amino acid change in the Cdk5-phosphorylation site on Munc18-1 (Milh et al., 2011), which is in detail discussed in *Chapter 4*. In this patient T574 is substituted by a proline residue resulting in seizures from the first day of life until the age of 1. At the age of the patient was free of seizures, but still displayed tremor, jerks stereotypic movements and ataxic walk. Furthermore, the patient showed autistic features and was unable to speak (Milh et al., 2011). Due to the mutation, Cdk5 cannot phosphorylate Munc18 at T574 and it is tempting to speculate that this lack of modulation underlies disease etiology. Future research in our lab will address this hypothesis.

Genomics, Proteomics, what's next? A perspective

Undoubtedly, the fully sequenced human genome in 2003 and the generation of the first knockout mouse already 22 years ago have greatly changed science and significantly contributed to our understanding of gene functioning. As a next step, proteomics aims to further unravel the puzzle by identifying protein interactomes in different subcellular compartments. However, the situation is more complex than initially thought. A plethora of posttranslational modifications adds an extra layer of complexity that allows for near endless combinations. Firstly, there is intensive crosstalk between different signaling cascades. Especially kinase pathways with multiple kinases in sequence such as the ERK pathway allow for regulation on different levels. For instance, Cdk5 modulates ERK activity by phosphorylation and inhibition of the upstream kinase MEK (Zheng et al., 2007).
Secondly, multiple posttranslational modifications can occur on a single protein. On the same target proteins two (or more) posttranslational modifications can work synergistically, i.e. one posttranslational modification leads to a second type of posttranslational modification, or



Posttranslational modifications of Munc18-1

S313 and S306 phosphorylations are mutually exclusive.

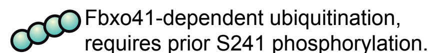


Figure 6.5 Combinatorial posttranslational modifications of Munc18-1. Munc18-1 can be phosphorylated by PKC at S306 or S313 (blue), ERK at S241 (red) and Cdk5 at T574 (green). Munc18-1 is ubiquitinated by Fbxo41 after prior ERK phosphorylation. Already the combination of only these 4 posttranslational modifications allows for 18 different Munc18-1 variants.

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antagonistically, i.e. one posttranslational modification prevents another type of posttranslational modification. *Chapter 3* of this thesis identifies a pathway in which ERK phosphorylation triggers Fbxo41-dependent ubiquitination of Munc18-1. Several other examples of interaction between phosphorylation and ubiquitination have recently been described in neurons (Bianchetta et al., 2011; Pak and Sheng, 2003; Seeburg et al., 2008). For Munc18-1, three kinases have been identified that phosphorylate the protein; ERK (*Chapter 3*), Cdk5 (*Chapter 4*) and PKC (Fujita et al., 1996; Wierda et al., 2007) and based on phospho-peptides identified in proteomic screens additional kinases likely exist. In addition, Munc18-1 can be ubiquitinated by Fbxo41 only after prior phosphorylation by ERK (*Chapter 3*). Figure 6.5 illustrates the combinatorial power of posttranslational modifications on Munc18-1. Only with the posttranslational modifications described, already 18 different Munc18-1 species can be distinguished. It is currently not known, whether all these species exist in a cell at all times or whether some PTMs exclude others.

Therefore we need to change the way we address proteins, as each single protein can exist in many different capacities depending on the time and place of expression. Current (phospho)proteomics methods already allow identification of specific amino acids that are posttranslationally modified. However, with the widely used “bottom-up proteomics” in which all proteins are digested into small peptides it is not possible to identify which modifications take place simultaneously under certain conditions and how many species of a protein exist. Further development of “top-down proteomics” techniques that allow analysis of modifications in intact proteins is needed to allow high-throughput characterization of isoforms and posttranslational modifications (Tipton et al., 2011; Tran et al., 2011). Analysis of samples at different time points during behavioral paradigms will allow temporal resolution and cellular fractionations allow crude subcellular analysis. However, single synapse resolution cannot be obtained using proteomics. To this end, we need to look at the localization of posttranslationally modified proteins. Currently, protein phosphorylation is studied with phospho-specific antibodies, which are well suited for Western Blotting but are often not specific enough for immunofluorescence labeling. Ideally, fluorescent tags that visualize posttranslational events should be engineered that allow for single synapse detection of these

events. Clearly such a invention would boost the field in a same way as the identification of pH-sensitive EGFPs in the late ‘90s (Miesenböck et al., 1998). Genetic engineering of fluorescent reporters have been become increasingly successful in recent years and might tackle such challenges in the near future. Understanding isoform and posttranslational modification diversity is vital to unravel mechanisms that underlie the fine-tuning of synapses and synapse-specific changes in synaptic strength.