Materials & Methods

7

Reagents

Antibodies

Primary antibodies were purchased from the following companies and were used at the following concentrations (Table 7.1). For immunocytochemistry, Alexa-conjugated secondary antibodies (Molecular Probes) were used at 1:1000 dilutions. For Western Blots, alkaline phosphatase-conjugated goat-anti-rabbit or goat-anti-mouse immunoglobulins (Dako) were diluted 1:10000.

Drugs

Drugs were purchased from the following companies and used at the following concentrations (Table 7.2).

DNA constructs and Lentivirus production

Different Munc18-1 variants were generated using Quickchange (Stratagene) and subcloned into pSuper vector. We substituted amino acids at potential phosphorylation sites, e.g. S241 or T574, for alanines (A) to prevent phosphorylation or to aspartate (D) to mimic the phosphorylated state of Munc18-1.

For myc-tagged and flag-tagged Fbxo41 constructs, Fbxo41 was amplified by PCR from imageclone IRAVp968G01134D (imagenes) to create restriction sites for subcloning into pmyc291CDNA3 or pCMV3TAG1C (Invitrogen) plasmids, respectively.

For flag-tagged MAPK1 (ERK2) constructs, MAPK1 was amplified by PCR from imageclone IRAVp968B02100D6 (imagenes) to create restriction sites for subcloning into pCMV3TAG1C plasmid (Invitrogen). The HA-Ubi expression plasmid was a gift from Johan de Winter (Vrije Universiteit Amsterdam).

The liprin-α expression constructs and deletion mutants were generated by a PCR-based strategy using human liprin-α1 and human liprin-α2 cDNA (Serra-Pagès et al., 1998). and subcloned in pGW1-, pGW2- and pβactin-expression vectors (Kapitein et al., 2010). In bio-GFP-liprin-α2 fusions, a linker encoding the sequence MASGLNDIFEAQKIEWHEGGG, which is the substrate of biotin ligase BirA was inserted into the NheI and AgeI sites in front of the pEGFP-C2 (Clontech) and the liprin-α2 open reading frame subsequently subcloned in the biotin-tag-GFP vector.

Protein	Species	Type	Supplier	IF	WB
Actin	Ms	m	Millipore	---	1:10000
Bassoon	Ms	m	Stressgene	1:500	---
CASK	Ms	m	gift Hoogenraad Lab	1:100	---
ERK (p42/p44 MAPK)	Ms	m	Cell Signaling	---	1:1000
pERK (phospho p44/p42)	Rb	p	Cell Signaling	---	1:1000
Fbox41	Rb	p	SySy	1:500	1:500
flag	Ms	m	Sigma	1:1000	1:2000
GAPDH	Rb	p	Abcam	---	1:2000
GFP	Chi	p	Aves	1:1000	---
HA	Ms	m	Roche	1:500	1:1000
LAMP1	Ms	m	Stressgene	1:100	---
Liprin-α2	Rb	p	gift Hoogenraad Lab	1:250	3:1000
MAP2	Chi	p	Abcam	1:10000	---
MAP2	Ms	m	Chemicon	1:1000	---
Munc18	Rb	p	SySy	1:500	1:5000
Munc18	Ms	m	BD Biosciences	---	1:2000
pMunc18$_{S241}$	Rb	p	Phosphosolutions	N.A.	1:1000
Opa1	Ms	m	BD Biosciences	---	1:1000
Plk2	Rb	p	Abcam	---	1:1000
PSD-95	Ms	m	Abcam	1:250	---
Rab3	Rb	p	SySy	1:1000	---
RIM1	Ms	m	BD Biosciences	1:200	---
SynapsinI/II	Rb	p	SySy	1:1000	1:1000
pSynapsin	Rb	p	SySy	---	1:1000
Syntaxin	Rb	p	SySy	1:1000	1:1000
Syntaxin (HPC1)	Ms	m	Sigma	---	1:2000
Transferrin receptor (TfR)	Ms	m	Zymed	1:500	---
tubulin	Ms	m	SySy	---	1:5000
VAMP	Ms	m	SySy	1:2000	---
VGCC ($Ca_v2.1$)	Rb	p	SySy	1:1000	---

Table 7.1: Antibodies.
Dilutions and suppliers of primary antibodies for immunofluorescence staining (IF) and Western Blots (WB). Abbreviations: Ms: mouse; Rb: rabbit; Chi: chicken; p: polyclonal; m: monoclonal, N.A.: not applicable because antibody is not suitable for this technique; ---: not tested.

Drug	Function	Supplier	Solvent	conc.
BDNF	neurotrophin	Sigma	H_20	100ng/ml
Bicuculline	$GABA_A$ receptors antagonist	Ascent	H_20	40μM
Cycloheximide	Translation inhibitor	Sigma	DMSO	20μg/ml
D-AP5	NMDAR antagonist	Ascent	H_20	50μM
DNQX	Non-NMDAR GluR antagonist	Enzo	DMSO	10μM
GABAzine	$GABA_A$ receptors antagonist	Ascent	H_20	10mM
Glycine	NMDAR and glycine receptors agonist	Sigma	H_20	200mM
Lactacystin	Proteasome inhibitor	Sigma	H_20	10μM
LY379268	Highly selective group II mGluR agonist	Tocris	H_20	1μM
MG132	Proteasome inhibitor	Sigma	DMSO	20μM
PD98059	MEK inhibitor	Sigma	DMSO	10μM
Picrotoxin	$GABA_A$ receptors antagonist	Ascent	EtOH	100μM
PMA	Phorbolester, activates PKC	Sigma	DMSO	1μM
α-PMA	Inactive compound of PMA	Sigmap	DMSO	1μM
Strychnine Hydrochloride	Inhibits glycine receptors	Sigma	H_20	1μM
TTX	blocks Na+v channels, block APs	Ascent	H_20	2μM
WIN55,212-2	CB1R agonist	Sigma	H_20	5μM

Table 7.2: Drugs
Dilutions, solvent and suppliers of drugs. Abbreviations: H_20: water, EtOH: ethanol

Protein	shRNA target sequence	Species	Ref.
Liprin-α1 #1	5'-tctgtgcatgacctcaatg	R/Ms/H	Oligo Engine
Liprin-α2 #2	5'-agccagtctgattacagaa	R/Ms/H	Oligo Engine
Liprin-α2 #3	5'-gcatgaacttcttgaagaa	R/Ms/H	Oligo Engine
Fbxo41 ....#3	5'-gctgccctcttctgtatc	R/Ms/H	iRNAi
scrambled	5'-ttctccgaacgtgtcacgt	R/Ms/H	Zhang et al. (2008)
Bassoon	5'-aacacctgcacccagtgtcac	R/Ms/H	Atasoy et al. (2007)
CASK	5'-gcactgaatcacccatggc	R/Ms/H	Oligo Engine
RIM1	5'-agtccagacggtaaagttc	R/Ms/H	siRNA at Whitehead
Piccolo	5'-aagtgctgtctcctctgttgt	R/Ms	Atasoy et al. (2007)

Table 7.3: shRNA sequences
shRNA sequences used to knockdown synaptic proteins. Abbreviations: R: rat; Ms: mouse; H: human

For knockdown of proteins, shRNAs were designed in the iRNAi or the siRNA selection program at Oligo Engine to target the sequence depicted in Table 7.3 and were inserted into pSuper vector. For Lentiviral constructs, liprin-α1, liprin-α2, scrambled and Fbxo41 shRNA were inserted into pLentiLox3.7 vectors which contained EGFP driven by a human synapsin1 promoter (Rubinson et al., 2003).
All DNA constructs were verified by sequencing and subcloned into pLenti vectors, and viral particles were produced as described (Naldini et al., 1996). Before usage of the Lentivirus on neurons, virus titer was determined by infecting HEK293T cells (in serum containing medium) with different dilutions of Lentivirus (0.1 – 1µl range). Cells were fixed when confluence was reached (between 24-48h after transfection) and immunostained for the overexpressed protein. Infected cells were counted in confocal images of at least 15 fields of view (40x oil objective, 0.7 zoom). Transduction efficiencies were taken into account when viruses were applied to neuronal cultures. Generally, neurons were infected DIV0 (for Munc18 rescue) or DIV9 (on WT mouse or rat cells).

Lipofectamine transfection
Network cultures in *Chapter 2* were transfected using Lipofectamine 2000 (Invitrogen). Briefly, DNA (3.6 µg /well) was mixed with 3 µl Lipofectamine 2000 in 200 µl NB, incubated for 30 minutes and then added to the neurons in NB at 37°C in 5% CO_2 for 45 min. Next, neurons were washed with NB and transferred in the original medium at 37°C in 5% CO_2 for 2-4 days.

Animals

Munc18-1 *null* mutant mice were generated as described previously (Verhage et al., 2000). E18 embryos were obtained by caesarian section of pregnant females from timed heterozygous matings. Munc18-1 *null* mutant mice are stillborn and can be easily distinguished from wild type or heterozygous littermates. Newborn P_0-P_1 pups from pregnant female Wistar rat (Harlan or Charles River) were used for rat neuronal cultures and glia preparations. Animals were housed and bred according to Institutional and Dutch governmental guidelines.

Foot shock paradigm

Male 9 to 10-week-old C57BL/6N mice (Charles River) were divided randomly into 3 groups (no shock control (NS), immediate shock (IS) and delayed shock (DS); n=5 per group). Mice of the NS control group were placed in the conditioning chamber for 212 s without being subjected to an unconditioned stimulus (US; electrical stimulation). Mice receiving an immediate shock were placed in the conditioning chamber followed immediately by the onset of the US (0.7 mA, 2 s duration, constant current) and then remained for another 210 s in the conditioning chamber. For delayed shock, mice were placed in the conditioning chamber for 180 s before the onset of the US [same specifications as above] and thereafter remained in the conditioning chamber for another 30 s. After 212 s all mice were placed back into the home cage for 30min before being sacrificed by cervical dislocation. Hippocampi were extracted on ice and immediately frozen to -80°C until further use. On the basis of this temporal training sequence, only DS mice acquire conditioned contextual fear whereas IS mice serve as control for US effects (Stiedl et al., 2004; Wiltgen et al., 2001).

Cell culture

Glia preparation

Forebrains of newborn (P_0-P_1) rat pups were dissected and freed from meninges in ice-cold Hanks Buffered Salt Solution (HBSS, Sigma) buffered with 7mM HEPES (Invitrogen). After removal of the hippocampus, hemispheres were incubated in 20-25 U/ml papain enzyme solution for 60 min at 37°C. After washing, cells were triturated using a firepolished

Pasteur pipette. Cells from one animal were plated in pre-warmed DMEM medium (Invitrogen) supplemented 10% fetal calf serum (FCS), 1% non-essential amino acids (NEAA) and 1% penicillin/streptomycin (all Gibco) with in a T175 flasks. Medium was refreshed the next day. Cells were allowed to grow for 1 week at 37°C, 5%CO_2. Single-cell suspensions were obtained with Trypsin-EDTA (Sigma).

For *cultures containing a glia feeder layer*, cells were frozen and stored in liquid nitrogen. Cells were allowed to recover and divide for 1 week after thawing before further use. Glass coverslips, disinfected with 96% ethanol, were sprayed with a mixture of 0.1mg/ml poly-D-lysine (Sigma), 0.2mg/ml rat tail collagen (BD Biosciences) solution and 10.2mM acetic acid solution (Sigma) and subsequently UV-sterilized for 20min. Astrocytes were plated at a density of 25k per ml. 4-5 days after plating, neurons were plated at a density of 25k per 12well-well or 100k per 6well-well. Neurons were allowed to grow for 14-18 days in supplemented Neurobasal and half of the medium was replaced at least once a week.

For autaptic physiology, *glia island cultures* were made only from non-passaged glia cells.18mm glass coverslips (Menzel) were put in porcelain racks on a shaker and were etched in 1M HCl for at least two hours. Subsequently, coverslips were neutralized with 1M NaOH for maximum one hour, washed thoroughly with MilliQ water and once with 70% Ethanol. Until usage, coverslips were stored in 96% Ethanol. 1.5mg/ml agarose type II-A (Sigma) was applied in a thin layer onto the coverslip with a cotton swop. On the day of glia plating, agarose-covered coverslips were stamped with mixture of 0.1mg/ml poly-D-lysine (Sigma), 0.2mg/ml rat tail collagen (BD Biosciences) and 17mM acetic acid (Sigma) using a custom made rubber stamp (dot diameter 250µm). Coverslips were UV sterilized for 20 min after stamping. Astrocytes were plated at a density of 6k per ml and were allowed to form microislands for 4-5 days. Isolated hippocampal neurons were plated on astrocyte microislands at a density of 2k per well (rat) or 6k per well (Munc18 *null* mutants) and were grown in supplemented Neurobasal. Only islands containing single neurons were examined at DIV14-17.

For *cultures without astrocytes*, glass coverslips, disinfected with 96% ethanol, or culture dishes were coated with a mixture of 0.0005% poly-L-ornithine (Sigma) and 2µg/ml laminin (Sigma) or 30 µg/ml poly-L-lysine and 2 µg/ml laminin in PBS overnight and subsequently washed 3 times with

Plate	Type	Glia density	Genotype neurons	Neurons density
12-well	Island	6k/well	WT mouse	2k/well
			Munc18 KO	6k/well
			rat	2k/well
	Astrocyte feeder layer	25k/well	WT mouse	25k/well
	On glass	---	Munc18 KO	75k/well
			rat	75k/well
6-well	Astrocyte feeder layer	50k/wel	rat	100k/well
	On glass	---	Munc18 KO	150k/well

Table 7.4: Plating densities of glia cells and neurons for different types of cell cultures.

water for at least 5 min. Culture dishes were then filled with supplemented Neurobasal and stored at 37C, 5%CO_2 until further use. Neurons were plated at a density of 150k per 6well-well and allowed to grow for 11 days before further use.

Dissociated neurons

Hippocampi and cortices were separately collected in ice-cold Hanks Buffered Salt Solution (HBSS; Sigma) buffered with 7 mM HEPES (Invitrogen). After removal of the meninges, neurons were incubated in Hanks-HEPES with 0.25% trypsin (Invitrogen) for 20 minutes at 37°C. After washing, neurons were triturated using a fire-polished Pasteur pipette and counted in a Fuchs-Rosenthal chamber. Neurons were plated in pre-warmed Neurobasal medium (Invitrogen) supplemented with 2% B-27 (Invitrogen), 1.8% HEPES, 0.25% glutamax (Invitrogen) and 0.1% Pen/Strep (Invitrogen) at the following densities (Table 7.4)

Heterologous cell culture and transfection

HEK293T cells were cultured in DMEM medium containing 10% FCS and 1% penicillin/streptomycin. For biochemistry, HEK293T cells were plated at equal density in 10 cm dishes two days before transfection. Cells were transfected with calcium phosphate transfection at 80% confluence and grown for 24 hours after transfection before harvesting for Western Blot or immunocytochemistry.

Acute brain slices

After decapitation of adult (>8 weeks) wild-type mice, coronal brain slices (300µm) were sectioned in ice-cold solution containing: 110mM Choline chloride, 11.6mM Na-ascorbate, 7mM $MgCl_{2,}$ 3.1mM Na-pyruvate, 2.5mM KCl, 1.25mM NaH_2PO_4, 0.5mM $CaCl_2$, 26mM $NaHCO_3$, 10mM glucose (carboxygenated with 5% $CO_{2/}$95% O_2). Slices were allowed to recover for 1 hour in ACSF (125mM NaCl, 3mM KCl, 1.2mM NaH_2PO_4, 1mM $MgCl_2$, 2mM $CaCl_2$, 26mM $NaHCO_3$, 10mM glucose; carboxygenated with 5% CO_2/95% $O_{2)}$ at 37°C before application of the drugs. Drugs were diluted in ACSF to end concentration (see Table 7.2) and applied for 4 hours (unless otherwise stated) at 37°C. Slices were transferred on ice for further biochemical processing (see cellular fractionation).

Electrophysiology

Whole cell voltage-clamp (V_m=-70mV) recordings were acquired with an Axopatch 200A amplifier (Axon Instruments), Digidata 1322A and Clampex 9.0 software. Recordings were performed at DIV14-17 with boro-cillicate glass pipettes (2.5-4 mOhm) containing (in mM): 125 K^+-gluconate, 10 NaCl, 4.6 $MgCl_2$, 4 K_2-ATP, 1 EGTA, 15 Creatine Phosphate, 20 U/ml phosphocreatine kinase (pH 7.30, 300 mOsm). The external medium contained (in mM): 140 NaCl, 2.4 KCl, 4 $CaCl_2$, 4 $MgCl_2$, 10 HEPES, 10 glucose (pH 7.30; 300 mOsm). Action potentials were induced by 0.5ms depolarizing steps to +30 mV. Hypertonic sucrose (500mM) was applied to assess RRP size. Sucrose and all other drugs were applied using a fast barrel application system (Perfusion Fast-Step, Warner Instruments) and contained amaranth for visualization of the bath flow. P_{ves} was calculated as the response to a single stimulus divided by the response to hypertonic sucrose. All recordings were performed at room temperature. Custom made Matlab routines, Clampfit 9.0 and Minianalysis software was used for offline analysis.

Yeast two hybrid system

Yeast two-hybrid studies were generally performed using the MATCHMAKER Two-Hybrid System 3 (Clontech Laboratories, Palo Alto, CA) according to protocols of the manufacturer. Briefly, Munc18-1 fused to GAL4 DNA-binding domain was used as bait. Empty GAL4 was used as negative control bait. As prey, ERK1 or ERK2 cDNA was fused to the GAL4 transcription activation domain. When bait and prey proteins interact, the DNA-binding domain and activation domain are brought into proximity, thus activating transcription of the reporter genes ADE2 and HIS3. Yeast growth selection on synthetic dropout media (-ADE or –HIS) was performed in strict accord with the manufacturer's protocol.

Biochemistry

Brain extracts and fractionations

For preparations of brain lysate to be used for immunoprecipitation, brains were pottered in hypotonic PKB buffer (50mM Tris pH7.5, 1.5mM $MgCl_2$, 5.0mM EDTA, 100mM NaCl; 2 embryonic brains in 3ml or 1 adult brain in 6ml) containing protease inhibitors. Next Triton-X100 (final concentration 1%) and NaCl (final concentration 150mM) were added and samples tumbled for 30min at 4ºC. For whole cell protein level analysis, brains were pottered in PBS (pH7.40) and subsequently centrifuged for 1min. The obtained pellet was re-suspended in 1x Laemmli Sample Buffer (LSB; containing 2% SDS, 10% glycerol, 0.26M β-mercaptoethanol, 60mM Tris pH6.8) by vortexing and boiling. DNA was sheared using a syringe needle until sample was liquid.

For preparation of crude synaptosomal fraction (P2; including synaptosomes, mitochondria and myelin), hippocampi were homogenized with an electrical potter (12 strokes at 900rpm) in homogenization buffer (5mM HEPES/NaOH pH7.4, including 350mM sucrose, protease inhibitor (Sigma) and phosphatase inhibitor cocktail 2 and 3 (Sigma)). To separate cell debris and nuclei, homogenates were pelleted at 1000g for 10min at 4ºC. Supernatants were pelleted again at 20000g for 30min at 4ºC to obtain P2 fractions.

For hippocampal P2 fractions from the fear paradigm, the obtained pellets were re-suspended in buffer containing 5mM HEPES/NaOH, pH

7.4, including 350mM sucrose, 150mM NaCl, protease and phosphatase inhibitors. Protein concentration was determined by Bradford protein assay (BIO-RAD) and 20µg protein per condition was subjected to Western Blot analysis.

For slice P2 fractions from drug application experiments, the obtained pellets were re-suspended in 1% deoxycholate (DOC) lysis buffer (150 mm NaCl, 50 mm Tris, pH 8.8, 1% DOC) and lysed for 30min. Subsequently, an equal volume of modified RIPA (dilution buffer) was added (150 mm NaCl, 50 mm Tris/HCl, pH 7.4, 1 mm EDTA, 1% Triton X-100, 0.1% SDS) (Kalia et al., 2006). All buffers were supplemented with protease and phosphatase inhibitor cocktails (Sigma). Protein concentration was determined by Bradford protein assay (BIO-RAD).

Immunoprecipitation and Western Blots

For immunoprecipitations from slice P2 fractions from drug application experiments 500mg of protein was incubated together with primary antibody and 75ml of 10% Protein A beads in PBS overnight. The next day, beads were washed 3 times with a 1:1 mixture of lysis and dilution buffer (see above). Protein were eluted by boiling in 2x Laemmli sample buffer and analyzed by Western Blotting.

For experiments in HEK cells or neuronal cultures, cells were lysed in either Laemmli Sample Buffer (for Western blot or denatured immunoprecipitation, containing 2% SDS, 10% glycerol, 0.26M β-mercaptoethanol, 60mM Tris pH6.8) or PKB buffer (for co-immunoprecipitation, containing 50mM Tris pH7.5, 1% Triton-X100, 1.5mM MgCl2, 5.0mM EDTA, 100mM NaCl). Immunoprecipitations were performed for at least 2 hours or overnight at 4ºC using antibodies conjugated to Protein A Agarose beads (Sigma). Samples were washed 4 times with buffer, proteins were eluted by boiling in 1x Laemmli sample buffer and analyzed by Western blotting.

For biotinylated protein pull-down assays, Munc18 variants with biotinylation (bio) tag were transfected together with the E. coli BirA biotin protein ligase in HEK-293T cells. Protein complexes of biotinylated Munc18 were subsequently pulled-down with streptavidin-coated paramagnetic beads (Magnaselect, Sigma) washed in low salt wash buffer (50mM Tris pH7.5, 1% Triton-X100, 1.5mM MgCl2, 5.0mM EDTA, 100mM NaCl) and high salt wash buffer (50mM Tris pH7.5, 1% Triton-X100, 1.5mM MgCl2, 5.0mM EDTA, 200mM NaCl) in the following sequence: low, high, low,

high, low. Proteins were eluted by boiling in 1x Laemmli sample buffer and analyzed by Western blotting.
For Western Blots, samples were loaded into 8-10% SDS-PAGE gels and run at 30mA per gel until satisfactory mass separation. Proteins were then transferred to PVDF membranes or in case of phospho-proteins on nitrocellulose membrane at 350mA. Blocking with 2% milk + 0.5% BSA or in case of phospho-proteins 2% BSA for at least 1 hour was used to circumvent unspecific binding. Primary antibodies were applied 2 hours or over night at 4ºC. After substantial washing, alkaline phosphatase labeled secondary antibodies were applied for 1 hour at 4ºC. Blots were then washed and scanned using ECF substrate for Western Blot (GE Healthcare) on a Fujifilm FLA-5000 Reader. All solutions for blocking, staining or washing were prepared in PBS (pH 7.40) containing 0.1% Tween-20 or in case of phospho-proteins in TBS (pH7.40) containing 0.1% Tween-20. Western Blots were stripped using Re-blot Plus Strong Antibody Stripping Solution (Millipore). Results were analyzed using the GelAnalyzer tool in ImageJ (NIH; Bethesda, MD).

***In vitro* kinase assay**
Different Munc18-1 constructs were transfected into HEK cells using calcium phosphate transfection. Cells were lysed in PKB buffer. After Munc18-1 immunoprecipitation (see above) samples were washed 3 times with PKB buffer and 2 times with kinase buffer (Cell Signaling). To each sample, 20µl kinase buffer containing (radioactively labeled) ATP and kinase was added and incubated for 30min at 37ºC. The reaction was stopped by addition of 5x LSB. Samples were analyzed using Western Blot.

Mass spectrometry
For mass spectrometry of liprin interactors, brains were harvested from adult female rats and P2 fractions were isolated as described (Lee et al., 2001) and extracted with 1% Sodium Deoxycholate (DOC) in 500mM Tris (pH 9.0) by incubation for 30 minutes at 36ºC. Extracts were dialyzed overnight into a 50mM Tris (pH 7.4)/0.1% Triton X-100 solution and spun down at 13,200 rpm for 40 minutes. The resulting supernatant is referred to as the P2/DOC extract and was incubated with the Dynabeads containing bio-liprin-α2 or bio-GFP for 2 hours at 4ºC and washed with low salt wash

buffer for three times. For protein elution, the beads were boiled in NuPAGE LDS 4 sample buffer (Invitrogen), separated, and supernatants were run on a 4-12% NuPAGE Tris-acetate gel (Invitrogen). The gel was stained with the Colloidal Blue staining kit (Invitrogen). For mass spectrometry analysis, 1D SDS-PAGE gel lanes were cut into 2-mm slices using an automatic gel slicer and subjected to in gel reduction with dithiothreitol, alkylation with iodoacetamide and digestion with trypsin (Promega, sequencing grade), essentially as described previously (Jaworski et al., 2009). Nanoflow LCMS/MS was performed on an 1100 series capillary LC system (Agilent Technologies) coupled to an LTQ linear ion trap mass spectrometer (Thermo) operating in positive mode and equipped with a nanospray source. Peptide mixtures were trapped on a ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 1.5 cm × 100 µm, packed in-house) at a flow rate of 8 µl/min. Peptide separation was performed on ReproSil C18 reversed phase column (Dr Maisch GmbH; column dimensions 15 cm × 50 µm, packed inhouse) using a linear gradient from 0 to 80% B (A = 0.1 M acetic acid; B = 80% (v/v) acetonitrile, 0.1 M acetic acid) in 70 min and at a constant flow rate of 200 nl/min using a splitter. The column eluent was directly sprayed into the ESI source of the mass spectrometer. Mass spectra were acquired in continuum mode; fragmentation of the 4 peptides was performed in data-dependent mode. Peak lists were automatically created from raw data files using the Mascot Distiller software (version 2.1; MatrixScience). The Mascot search algorithm (version 2.2) was used for searching against the International Protein Index database (release number IPI_rat_20100507.fasta or IPI_human_20100507.fasta). The peptide tolerance was typically set to 2 Da and the fragment ion tolerance to 0.8 Da. A maximum number of 2 missed cleavages by trypsin were allowed and carbamidomethylated cysteine and oxidized methionine were set as fixed and variable modifications, respectively. The Mascot score cut-off value for a positive protein hit was set to 60. Individual peptide MS/MS spectra with Mowse scores below 40 were checked manually and either interpreted as valid identifications or discarded. Proteins present in the negative controls (pull-down assays with bioGFP alone) were omitted from the table.

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Imaging

Immunocytochemistry

Cells were fixed in 2% formaldehyde (Electron Microscopy Sciences), then 4% formaldehyde (10 minutes each at room temperature). Cells were permeabilized in 0.5% Triton X-100/PBS for 5 min and blocked for 30 min in 2% normal goat serum/0.1% Triton X-100/PBS. Antibodies were diluted in blocking solution and applied for 1 hour at room temperature. After washing with PBS, cells were incubated with Alexa-conjugated secondary antibodies (Invitrogen) for 1 hour at room temperature.

Alternatively, cells were incubated with primary antibodies in GDB buffer (0.2% BSA, 0.8 M NaCl, 0.5% Triton X-100, 30 mM phosphate buffer, pH 7.4) overnight at 4ºC. Neurons were then washed three times in PBS for 5 min at room temperature and incubated with Alexa-conjugated secondary antibodies in GDB for 2 hours at room temperature and washed three times in PBS for 5 min.

In both cases, slides were mounted using DABCO-Mowiol (Invitrogen) to prevent fading of the fluorophore. All pictures were captured on a Zeiss LSM 510 confocal microscope. Unless otherwise stated, images were acquired with a 40x oil objective (NA = 1.3) and 0.7x mechanical zoom. For autaptic cultures, only a single plane was acquired. For network cultures, each image was a z-series of 10-12 images; each averaged 2 times and was chosen to cover the entire region of interest from top to bottom. The resulting z-stack was ‘‘flattened’’ into a single image using maximum projection. Images were not further processed and were of similar high quality to the original single planes. Confocal settings were kept the same for all scans when fluorescence intensity was compared.

Imaging of brain slices

For Figure 5.6 I, a layer 2/3 pyramidal neuron from a 300µm slice of the barrel region of the somatosensory cortex of an 8 week old mouse was filled with biocytin (0.2%) using an intracellular patch-clamp recording pipette. The slice preparation was fixed in paraformaldehyde in PBS and then processed for staining with the chromogen 3,3’diaminobenzidine tetrahydrochloride (DAB) using the avidin–biotin–peroxidase method (Horikawa and Armstrong, 1988). For Figure 5.6 K, a layer 2/3 pyramidal neuron from a 300µm medial prefrontal cortex coronal brain slice of a

P14 mouse was filled with Alexa 594 (40μM, Molecular Probes) via an intracellular patch pipette. The dye was allowed to diffuse for 20 minutes before the pipette was withdrawn, causing the somatic membrane to reseal. The neuron was then imaged using a LEICA RS2 two-photon laser scanning microscope with a 63x objective and Ti:Sapphire laser tuned to 840nm excitation. Z-stacks were taken using 1μm Z step intervals of overlapping regions of the neuron. These images were then stitched together and Z-compressed using Image J (NIH) software.

Image analysis and quantification

Images were analyzed in a semi-automated fashion using the Matlab based software routine SynD (Schmitz et al., 2011b). For further information on the software, see *Chapter 5*.

Alternatively, MetaMorph software (Universal Imaging Corporation) was used to do the following analysis in *Chapter 2* on network cultures:

Morphometric analyses of hippocampal neurons. Synapse size in dissociated hippocampal neurons was determined by co-transfection of soluble mCherry to fill the cell. The average pixel length and height per synapse were measured using MetaMorph software on the z-section where the synapse appeared largest.

Quantification of fluorescent intensity: Images of dissociated hippocampal neurons were analyzed using MetaMorph software (Universal Imaging Corporation). Presynaptic boutons were morphologically identified as swellings along GFP labeled axon segments as described (Bamji et al., 2003; Leal-Ortiz et al., 2008) or by co-localization with PSD-95 and bassoon. Fluorescence intensities were measured using confocal images taken with identical laser power and microscope settings. A region was drawn around each synapse and the average intensity within the region was measured, corrected for background staining, and normalized to non-transfected neurons in the same field. Images of autaptic neurons were analyzed in Matlab with SynD (Schmitz et al., 2011a). Dendrites were detected based on the staining for MAP2 or GFP expression. Synapses were detected based on the staining for VAMP or Synapsin. Detected synapses mask was used to measure the synaptic intensity of additional proteins. Measurements of FM4-64 recycling at presynaptic boutons were performed as described (Murthy et al., 1997). Synapses from >5 neurons per experiment were identified in MetaMorph by morphology and overlaid

onto the FM4-64 images for quantification of fluorescence intensity using MetaMorph software. The intensity of all points following unloading was normalized to the intensity of that synapse prior to unloading. Separate experiments were adjusted to control to allow for comparison and analyzed in a blind manner. Statistical significance was measured by ANOVA repeated measurements in SPSS 16.0 (SPSS, Inc.).

Time-lapse live imaging, SV exocytosis and FRAP experiments

Time-lapse live cell imaging in *Chapter 2* was performed on an inverted research microscope Nikon Eclipse TE2000E (Nikon) with a CFI Apo TIRF 100x 1.49 N.A. oil objective (Nikon), equipped with a Coolsnap and a QuantEM EMCCD cameras (Roper Scientific) controlled by MetaMorph 7.1 software (Molecular Devices). For SV exocytosis experiments, we used a HBO 103 W/2 Mercury Short Arc Lamp (Osram) and a Chroma ETGFP/mCherry filter cube for excitation. To separate emissions we used DualView (Optical Insight) with emitters HQ530/30M and HQ630/50M (Chroma) and the beam splitter 565DCXR (Chroma). For FRAP experiments, cells were imaged with Total Internal Reflection Microscopy (TIRF) using a 488 nm laser. During imaging cells were maintained at 37ºC in either Tyrode solution (2.5mM KCl, 119mM NaCl, 25mM HEPES, 30mM Glucose, 2mM $CaCl_2$, 2mM $MgCl_2$), for SV exocytosis experiments, or the standard culture medium, for FRAP experiments, in a closed chamber with 5% CO_2 (Tokai Hit; INUG2-ZILCS-H2).

For SV exocytosis experiments, transfected neurons were loaded with 10 µM FM4-64FX (Invitrogen) as described (Fernandez-Alfonso and Ryan, 2004) and areas containing transfected axons were selected. One image of the FM4-64-loaded synapses was taken prior to the second stimulation, and images were acquired at an interval of ~7 seconds for two minutes immediately following the second stimulation.

FRAP experiments were performed using the FRAP scanning head 3 FRAP L5 D – CURIE (Curie Institute) and the 488nm laser line for bleaching. To analyze the recovery of fluorescence, areas including the bleached synapse were selected and background subtracted frame-by-frame by subtracting the average intensity of an empty, non-bleached area. Recovery R was then calculated as R = (I(t)- I(directly after bleaching))/(I(before bleaching)-I(directly after bleaching), with I denoting total synapse intensity. After normalization, the final recovery R_{final} for each

individual trace was determined as the level at the end of the recording (450s after bleaching). The immobile fraction was then calculated as $1\text{-}R_{final.}$ The recovery half-time was determined for each trace as the first time point where recovery reached the level corresponding to half the final recovery. Analysis was performed using MetaMorph software (Universal Imaging Corporation).

Electron Microscopy

Autaptic neurons were fixed at DIV14-16 for 1-2 hours at room temperature with 0.1 M cacodylate buffer/0.25mM$CaCl_2$/0.5mM $MgCl_2$ (pH 7.4) (de Wit et al., 2006; Wierda et al., 2007). As for electrophysiology and imaging, only glia islands containing a single neuron were used for analysis. After fixation, cells were washed three times for 5 min with 0.1 M cacodylate buffer (pH 7.4), post-fixed for 2 hours at room temperature with 1% Osmium tetroxide/1% Rutheniumcanide in bidest, washed and stained with 1% uranyl acetate for 15 min in the dark. Following dehydration through a series of increasing ethanol concentrations, cells were embedded in Epon and polymerized for 24 h at 60°C. After polymerization of the Epon, the coverslip was removed by alternately dipping it in liquid nitrogen and hot water. Cells of interest were selected by observing the flat Epon embedded cell monolayer under the light microscope, and mounted on pre-polymerized Epon blocks for thin sectioning. Ultrathin sections (~ 90 nm) were cut parallel to the cell monolayer and collected on single-slot, formvar-coated copper grids, and stained in uranyl acetate and lead citrate (Leica ultrasainer). Autaptic synapses were selected at low magnification using a JEOL 1010 electron microscope. All analyses were performed on single ultrathin sections of randomly selected synapses. The distribution of synaptic vesicles, total synaptic vesicle number and active zone length were measured on digital images of synapses taken at 100.000x magnification using a custom-made Matlab routine. The observer was blinded for the genotype. For all morphological analyses we selected only synapses with intact synaptic plasma membranes with a recognizable pre- and postsynaptic density and clear synaptic vesicle membranes. Docked synaptic vesicles had a distance of 0 nm from the synaptic vesicle membrane to the active zone membrane. The active zone membrane was recognized as a specialized part of the presynaptic plasma membrane that contained a clear presynaptic density.

Data representation and statistics

In all bar graphs, data is presented as mean values ± SEM. Statistical analysis was performed with SigmaPlot v11.0 (Systat Software) or Instat v3.05 software (GraphPad Software). Data samples were first tested for normality with the Kolmogorov and Smirnov test and for heterogeneity of variance with the method of Barlett. If data allowed an unpaired t-test (with Welch correction if required) was used. Alternatively, the non-parametric Mann-Whitney U test was used to test for statistical significance. For multiple groups, One-way ANOVA or the non-parametric Kruskal-Wallis Test was used. P-values below 0.05 are considered significant (* $p<0.05$, ** $p<0.01$, *** $p<0.001$).

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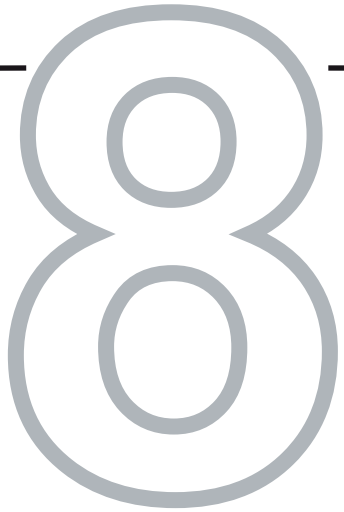

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Appendix

Appreviations

5-HT	5-hydroxytryptophan
Aβ	amyloid β
AD	Alzheimer's disease
AP	action potential
APC/C	anaphase promoting complex/cyclosome
AZ	active zone
BDNF	brain-derived neurotrophic factor
BRP	Bruchpilot
Ca^{2+}	calcium
CAZ	cytomatrix of the active zone
CB1R	cannabinoid receptor type 1
Cdk5	cyclin-dependent kinase 5
CN	calcineurin
ERK	extracellular signal-regulated kinase
FRAP	fluorescence recovery after photobleaching
FRET	Förster resonance energy transfer
H^+	proton
KD	knock-down
KI	knock-in
KO	knock-out
LTP	long-term potentiation
MAPK	mitogen-activated protein kinase
mGluR	metabotropic glutamate receptor
NMJ	neuromuscular junction
NT	neurotransmitter
PIS	pre-immune serum
PP	paired pulse
PPD	paired pulse depression
PPF	paired pulse facilitation
P_r	synaptic release probability
PTM	posttranslational modification
PTSD	post-traumatic stress disorder
P_{ves}	vesicular release probability
RRP	readily releasable pool
SM protein	Sec1/Munc18-like protein

SNARE	soluble N-ethylmaleimide-sensitive-factor attachment receptor
STXBP1	Syntaxin binding protein 1
SV	synaptic vesicle
SynD	synapse detector
THC	tetrahydrocannabinol
TrkB	tyrosine kinase receptor B
TRP	total release pool
WT	wild-type

Nederlandse samenvatting

Posttranslationele modificatie van synaptische sterkte

Bewegen, voelen, denken, leren en natuurlijk ook dit proefschrift lezen zijn maar een paar voorbeelden van dingen die wij met hulp van onze hersenen kunnen doen. Wetenschappers zijn al sinds eeuwen hierdoor gefascineerd en proberen erachter te komen hoe dit werkt. Technische ontwikkelingen zoals bijvoorbeeld de uitvinding van de microscoop leidde tot de overgang van beschrijvende anatomie naar microscopische en functionele analyse van het gezonde en zieke brein. Een belangrijke ontdekking maakte Ramón y Cajal aan het eind van de 19de eeuw toen hij de zenuwcel (het *neuron*) als functionele en structurele eenheid van het zenuwstelsel beschreef.

De menselijke hersenen bestaan uit ca. 100 miljard (10^{11}) zenuwcellen (Figure 1.1). Een typische zenuwcel heeft een cellichaam en vele uitlopers, de zogenaamde *dendrieten* en het *axon*. In onze hersenen vormen dendrieten en axonen in totaal ongeveer 100 biljoen (10^{14}) contact punten, de *synapsen*. Hierdoor zijn de hersenen met een soort supercomputer te vergelijken. Een enkele zenuwcel ontvangt informatie van vele anderen, integreert deze en geeft het resultaat door via zijn axon aan de dendrieten van andere zenuwcellen. Meestal zijn axon en dendriet niet direct met elkaar verbonden, maar door de *synaptische spleet* van elkaar gescheiden. Om deze afstand te overbruggen, gebruiken zenuwcellen chemische signaalstoffen (*neurotransmitters*) ter communicatie (Figure 1.2). De neurotransmitters worden in de eerste (presynaptische) cel in kleine blaasjes (*vesikels*) verpakt en in de buurt van het celmembraan bewaard. Zenuwcellen communiceren met elkaar als een elektrisch signaal (*actiepotentiaal*) calcium de presynaptische cel in laat stromen. Hier zitten eiwitten (*proteïnen*) die calcium kunnen herkennen en zo de fusie van de neurotransmitter blaasjes op gang brengen. Neurotransmitters worden dan in de synaptische spleet afgegeven en bereiken de tweede (postsynaptische) cel. Hier binden ze aan specifieke receptoren op het cel membraan en induceren het volgende elektrische signaal. Veel verschillende eiwitten zijn nodig om de verschillende stappen van deze synaptische cyclus te controleren, zodat de afgifte van neurotransmitter

altijd snel en precies kan plaatsvinden. Bovendien kan de sterkte van de neurotransmitter afgifte ook veranderd worden als er veranderingen in onze omgeving optreden. Denk hierbij bijvoorbeeld aan het leren van een nieuw onderwerp. Dit aanpassingsvermogen van de hersenen noemen wij *plasticiteit*.

Tijdens mijn promotietraject heb ik geprobeerd erachter te komen hoe dit op moleculair niveau gebeurt. Hiervoor heb ik met name naar de functie van twee synaptische eiwitten gekeken: liprin-α2 en Munc18-1. Verder heb ik gekeken hoe de functie van deze eiwitten aangepast wordt als de activiteit in de hersenen sterk verandert. Deze aanpassing gebeurt door *posttranslationele modificatie*, het toevoegen van kleine chemische groepen aan het eiwit.

Hoofdstuk 2 beschrijft de functie van de eiwitten liprin-α1 en liprin-α2 in de volwassen synaps. Wij laten zien dat liprin-α2, maar niet liprin-α1, belangrijk is voor de afgifte van neurotransmitter. Zonder liprin-α2 zijn de actieve zones, de plekken waar de afgifte plaats vindt, misvormd en zitten andere eiwitten niet op de goede plek. Bovendien beïnvloedt neuronale activiteit de hoeveelheid liprin-α2 die zich in de synaps bevindt door afbraak in het proteasoom. Hierdoor speelt liprin-α2 een belangrijke rol bij de aanpassing van de synaptische sterkte aan de hersenactiviteit.

Hoofdstuk 3 en 4 bestuderen de veranderingen aan het eiwit Munc18-1 door *fosforylering* (het toevoegen van een fosfaatgroep) door verschillende *kinasen* (eiwitten die fosfaatgroepen toevoegen).
Hoofdstuk 3 beschrijft een nieuwe, remmende functie van de ERK kinase op de afgifte van neurotransmitters. We laten zien dat ERK Munc18-1 fosforyleert als de neuronale activiteit hoog is, bijvoorbeeld tijdens stress bij een muis door een elektrische shock. Door de fosforylering wordt Munc18-1 door de E3 ligase eenheid Fbxo41 herkend en vervolgens geubiquitineerd en in het proteasoom afgebroken. Hierdoor ontstaat een verminderde afgifte van neurotransmitter. Het voorkomen van fosforylering leid to verhoogde afgifte en blokkeert het inhibitoire effect van cannabinoid (CB1) receptor activatie. Daardoor draagt de fosforylering van Munc18 door ERK bij aan de homeostatische controle van neuronale activiteit.

Hoofdstuk 4 bestudeerd het effect van Cdk5 fosforylering van Munc18-1. Door activatie van Cdk5 wordt de synaptische sterkte anders verdeeld, sommige synapsen worden zwakker, andere worden sterker (Mitra et al. 2011). Wij laten zien dat Cdk5 fosforylering van Munc18-1 belangrijk is voor de regulatie van synaptische sterkte. In afwezigheid van de fosforylering geven zenuwcellen minder neurotransmitter af. Hieruit kunnen we concluderen dat Cdk5 fosforylering van Munc18-1 niet bijdraagt aan het stilleggen van synapsen, maar dat het een belangrijke rol zou kunnen spelen bij de verhoging van synaptische sterkte tijdens lage neuronale activiteit.
Hoofdstuk 5 beschrijft een computer programma, *SynD*, dat voor de automatische analyse van neuronale morfologie gebruikt kan worden. Met het programma kunnen morfologische parameters en de intensiteitsmetingen uit het cellichaam, de uitlopers en de synapsen van immunofluorescente plaatjes van zenuwcellen uitgelezen worden. Bovendien gaat de analyse met het programma duidelijk sneller dan met de hand en ontstaan er minder problemen door observer bias.

Hoofdstuk 6 vat de hoofdbevindingen samen en probeert in een model uit te leggen hoe synaptische sterkte in de hersenen door middel van posttranslationele modificaties veranderd kan worden. Met name ligt hierbij de focus op liprin-α2 en Munc18-1 en de mogelijke implicaties voor de gezondheid van de mens. Het onderzoek in dit proefschrift laat zien dat een eiwit door veel andere eiwitten beïnvloed wordt en dat al kleine veranderingen zoals het toevoegen van een fosfaatgroep de functie van een eiwit sterk kunnen veranderen. Hierdoor kunnen al kleine afwijkingen in het eiwit zelf (bijvoorbeeld punt mutaties) of in de signaalcascade grote invloed hebben op het functioneren van ons brein. In het vervolg van dit onderzoek wordt gekeken in hoeverre de beschreven paden een rol spelen bij bijvoorbeeld de ziekte van Alzheimer, angststoornissen en epilepsie.

Deutsche Zusammenfassung

Posttranslationelle Modifizierung synaptischer Stärke

Bewegen, fühlen, denken, lernen - und natürlich das Lesen dieser Dissertation sind nur einige wenige Beispiele aus der schier endlosen Liste an Dingen, die unser Gehirn steuert. Seit Jahrhunderten sind Wissenschaftler davon fasziniert und versuchen herauszufinden, wie das Gehirn diese Aufgaben bewältigen kann. Technische Weiterentwicklungen wie beispielsweise die Erfindung des Mikroskops führten von rein deskriptiver Anatomie hin zu mikroskopischer und funktioneller Analyse des gesunden und kranken Gehirns. Ein Meilenstein gelang Santiago Ramón y Cajal am Ende des 19. Jahrhunderts mit der Beschreibung der Nervenzelle (*Neuron*) als funktionelle und strukturelle Einheit des Nervensystems.

Das menschliche Gehirn besteht aus circa 100 Milliarden (10^{11}) Nervenzellen (Abbildung 1.1). Eine typische Nervenzelle besteht aus einem Zellkörper und vielen Fortsätzen: multiplen *Dendriten* und einem *Axon*. Dendriten und Axone formen im Gehirn insgesamt circa 100 Billionen (10^{14}) Kontaktstellen (*Synapsen*), vergleichbar mit etwa einer Art Super- oder Quantencomputer. Eine einzige Nervenzelle emfängt Informationen von vielen anderen, integriert diese und leitet das Resultat über ihr Axon an die Dendriten anderer Nervenzellen weiter. Meist sind die Kontaktstellen von Axon und Dendriten nicht direkt miteinander verbunden, sondern durch einen kleinen *synaptischen Spalt* voneinander getrennt. Um diesen Abstand zu überwinden, verwenden Nervenzellen chemische Signalstoffe (*Neurotransmitter*) zur Kommunikation (Abbildung 1.2). Die Neurotransmitter werden in der ersten (präsynaptischen) Zelle in kleine Bläschen (*Vesikel*) verpackt und in der Nähe der Zellmembran aufbewahrt. Nervenzellen kommunizieren miteinander, wenn ein elektrisches Signal (*Aktionspotential*) den Einstrom von Kalziumionen in die präsynaptische Zelle auslöst. Die dort befindlichen Eiweissmoleküle (*Proteine*) erkennen das einströmende Kalzium und setzen so die Fusion der Bläschen mit der Zellmembran in Gang. Die so freigesetzten Neurotransmitter gelangen durch den synaptischen Spalt zur zweiten (postsynaptischen) Zelle. Dort binden sie an spezielle Rezeptoren auf der Zellmembran und lösen ein

weiteres elektrisches Signal aus. Viele verschiedene Proteine sind nötig, um die unzähligen kleinen Schritte dieses synaptischen Zyklus so zu kontrollieren, dass die Freisetzung von Signalstoffen schnell und exakt stattfinden kann. Um auf Veränderungen in unserer Umwelt reagieren zu können kann die Stärke der Neurotransmitterfreisetzung verändert werden - dies geschieht zum Beispiel beim Lernen. Dieses Anpassungsvermögen des Gehirns nennt man *Plastizität*.

In den letzten 4 Jahren habe ich unser Verständnis dafür erweitert wie diese Vorgänge auf molekularem Niveau ablaufen. In meiner Doktorarbeit untersuchte ich vor allem die Funktion von zwei Proteinen: Munc18-1 und Liprin-α2. Ausserdem habe ich den Einfluss neuronaler Aktivität auf die Funktion dieser Moleküle untersucht. Dies geschieht durch die Veränderung von chemischen Gruppen and der Proteinoberfläche, durch sogenannte *posttranslationelle Modifizierungen*.

Kapitel 2 untersucht die Rolle der synaptischen Proteine liprin-α1 und liprin-α2 bei der Freisetzung von Neurotransmittern. Wir zeigen, dass liprin-α2, aber nicht liprin-α1, wichtig ist für die Freisetzung von Neurotransmittern. Ohne liprin-α2 ist der Aufbau der aktiven Zone, der Stelle, an denen die Freisetzung stattfindet, fehlerhaft, und auch die Lokalisation weiterer Proteine ist beeinflusst. Außerdem beeinflusst neuronale Aktivität die Menge an liprin-α2, die sich in der Synapse befindet. Dies geschieht durch regulierten proteasomalen Abbau mit Hilfe der APC/C E3 Ligase. Daher spielt liprin-α2 eine wichtige Rolle in der Freisetzung von Neurotransmittern und Modulierung synaptischer Stärke im Gehirn.

Kapitel 3 und 4 untersuchen die Veränderungen des Proteins Munc18-1 durch *Phosphorylierungen* (dem Zufügen einer Phosphatgruppe an ein Protein) durch verschiedene *Kinasen* (phosphatgruppenübertragende Proteine).

Kapitel 3 beschreibt eine neue, hemmende Rolle der ERK Kinase in der Freisetzung von Neurotransmittern. Wir zeigen, dass Munc18-1 bei hoher neuronaler Aktivität durch ERK phosphoryliert wird. Dies geschieht in der Maus z.B. durch Stress, beispielsweise ausgelöst durch einen elektrischen Schock. Durch die Phosphorylierung wird Munc18-1 von der E3 Ligase Fbxo41 erkannt, anschliessend ubiquitiniert und im Proteasom abgebaut.

Hierdurch wird weniger Neurotransmitter freigesetzt. Das Verhindern der Phosphorylierung führt zu erhöhter Neurotransmitterfreisetzung und blockiert den hemmenden Effekt der CB1-Cannabinoidrezeptoraktivierung. Dadurch trägt die ERK-vermittelte Phosphorylierung von Munc18-1 zur homeostatischen Kontrolle neuronaler Aktivität bei.

Kapitel 4 untersucht den Effekt der Cdk5 Phosphorylierung von Munc18-1. Cdk5 Aktivität führt zu veränderter synaptischer Stärke. Einige Synapsen werden stärker, andere deutlich schwächer (Mitra et al. 2011). Wir zeigen, dass Cdk5 Phosphorylierung von Munc18-1 für die Regulation synaptischer Stärke wichtig ist. Ohne diese Phosphorylierung zeigen Nervenzellen eine verminderte Neurotransmitterfreisetzung. Hieraus können wir schliessen, dass Cdk5 Phosphorylierung von Munc18 nicht dazu beiträgt Synapsen still zu legen, sondern dass es eine wichtige Rolle beim Erhöhen der synaptischen Stärke in Zeiten niedriger neuronaler Aktivität spielen könnte.

Kapitel 5 beschreibt ein Computerprogramm, *SynD*, das für die automatische Analyse neuronaler Morphologie benutzt werden kann. Mit dem Programm können morphologische Parameter und Intensitätsdaten in Zellkörper, Synapsen und Fortsätzen aus Immunofluoreszenzbildern ausgelesen werden. Die Analyse mit SynD ist deutlich schneller als herkömmliche Methoden und weniger durch den Beobachter beeinflusst.

Kapitel 6 fasst die Hauptergebnisse zusammen und versucht in einem Modell zu erklären wie synaptische Stärke im Gehirn mit Hilfe von posttranslationalen Modifizierungen verändert werden kann. Hierbei liegt der Schwerpunkt hauptsächlich auf den Proteinen liprin-α2 und Munc18-1 und ihren möglichen Implikationen für die Gesundheit des Menschen. Diese Doktorarbeit zeigt, dass ein einziges Protein durch viele andere Proteine beeinflusst werden kann und dass selbst kleinste Veränderungen, wie z.B. das Hinzufügen einer Phosphatgruppe, die Funktion eines Proteins stark beeinflussen können. Hierdurch können schon kleine Veränderungen im Protein selbst (z.B. Punktmutationen) oder in Signalkaskaden unser Gehirn stark beeinflussen. In anschliessenden Forschungsprojekten wird nun untersucht inwiefern die beschriebenen Kaskaden eine Rolle bei beispielsweise Alzheimer, Angststörungen oder Epilepsie spielen können.

Curriculum vitae

Sabine Katharina Schmitz was born on 21st of June 1983 in Wittlich, Germany. She finished her secondary education in 2002 at the Peter-Wust-Gymnasium in Wittlich. After graduating from high school Sabine did internships at the Max-Planck-Institute for Neurological Research in Cologne and in the Department of Neurology at the St. Elisabeth Hospital Wittlich to find out whether her passion lies in medicine or basic science. Her interest in Neuroscience was born and that year she started to study Human Biology/Theoretical Medicine at the Philipps University in Marburg. During this time she was elected fellow of the German National Academic Foundation. In 2005, Sabine moved to Amsterdam to enter the Master of Neuroscience program at the Vrije Universiteit from which she graduated in 2007. In 2006, she carried out a project in the Department of Functional Genomics under the supervision of Dr. Ruud Toonen. In this year, Sabine also took part in the Exchange Honours Master of Neuroscience, a joined program between the Erasmus Medical Center Rotterdam and the Vrije Universiteit. In 2007, she moved to London to perform her second internship in the laboratory of Prof. Yukiko Goda at University College London. After this, she was awarded a Toptalent fellowship of the Nederlandse Wetenschapelijke Organisatie (NWO) and a Marie Curie Early Stage Training fellowship from the European Union to start her PhD research back in the Netherlands. From 2007 until 2011 she conducted her research in the Department of Functional Genomics at the Vrije Universiteit Amsterdam under the supervision of Dr. Ruud Toonen and Prof. Matthijs Verhage, which resulted in the current dissertation.

List of publications

Automated analysis of neuronal morphology, synapse number and synaptic recruitment.
Schmitz SK*, Hjorth JJ*, Joemai RM, Wijntjes R, Eigenraam S, de Bruijn P, Georgiou C, de Jong APH, van Ooyen A, Verhage M, Cornelisse LN, Toonen RF, Veldkamp WJ.
J Neurosci Methods. 2011 Feb 15;195(2):185-93

Dendritic position is a major determinant of presynaptic strength.
De Jong APH Schmitz SK, Toonen RF, Verhage M
accepted at Journal of Cell Biology

Liprin-α2 organizes presynaptic composition upstream of CASK and RIM to facilitate synaptic transmission
Schmitz SK*, Spangler SA*, de Graaff E, Demmers J, de Wit H, Toonen RF, Hoogenraad CC
submitted to Neuron

Munc13 controls the location and speed of neuronal dense core vesicle release
van de Bospoort R, Farina M, de Jong APH, Schmitz SK, de Wit H, Verhage M, Toonen RF
submitted to EMBO

ERK-dependent recruitment of Fbxo41 degrades Munc18-1 and controls synaptic strength
Schmitz SK, King C, Saarloos I, Kevenaar J, de Wit H, Stiedl O, Li KW, Smit AB, Verhage M, Toonen RF
in preparation

Cdk5 phosphorylation of Munc18-1 enhances synaptic transmission
Schmitz SK, Verhage M, Toonen RF
in preparation

Dankwoord

Het is vandaag 09 maart 2012, het regent, het is koud en ik zit op ruim 3500m hoogte in mijn tent onderweg naar Machu Picchu. Drie dagen geleden hoorde ik dat de commissie mijn proefschrift goedgekeurd had. *Het is af! Eindelijk! Gelukkig! Ongelofelijk!* Maar er mist nog iets heel belangrijks: het DANKWOORD! Laat ik nu maar beginnen om al die mensen te bedanken die door hun hulp, steun en gezelligheid in de afgelopen 4 jaar aan dit boekje en mijn AIO tijd bijgedragen hebben.

Ik wil beginnen met mijn begeleiders, Ruud en Matthijs. Het begon allemaal in 2005 toen ik naar Nederland kwam. Al snel hebben jullie mij een AIO plek aangeboden. Een grote verrassing, want eigenlijk wou ik maar een jaar in Nederland blijven. Maar zo te zien, ben ik er nog steeds en waren mijn plannen snel veranderd. Ruud en Matthijs, het verschil in jullie begeleiding had niet groter kunnen zijn en die combinatie maakte het juist sterk. Ruud, ik was jouw eerste AIO en besef eigenlijk nu pas dat dat best een risico had kunnen zijn. Ik ben blij dat ik ervoor gekozen heb. Je deur en mailbox staan 24/7 open voor je AIOs en je enthousiasme voor wetenschap en motivatie zijn indrukwekkend. Matthijs, bedankt voor je inzicht in het grote geheel en je voortdurende interesse in mijn project. Samengevat zou je dus kunnen zeggen: de ‘grote’ baas voor het grote plaatje en de ‘kleine’ baas voor alle details. Allebei bedankt voor de vrijheid die ik in mijn project had en de kansen die jullie mij gegeven hebben om mijn horizon te verbreden.

Verder wil ik mijn collega’s van de afdeling Functional Genomics (FGA) bedanken voor alle zinnige discussies en adviezen, dagelijkse pret en gezelligheid binnen en buiten het lab. Een aantal wil ik hier apart noemen:

Cillian, je bent begonnen als mijn eerste student toen we het project nog aan het opstarten waren. Gelukkig heeft je dat niet afgeschrikt en ben je ook als AIO bij ons komen werken. Sindsdien gaan we als stERK team aan de slag. Ik vond het leuk om met jou samen te werken en ben trots op onze resultaten in hoofdstuk 3.

Ingrid, bedankt voor de duizenden blotjes en je kritische blik op de soms wilde ideeën van Ruud en mij. En mocht je ooit geen zin meer in wetenschap hebben, moet je misschien eens over een carrière in “matchmaking” gaan nadenken ;)

Natuurlijk wil ik ook mijn kamergenootjes uit het kippenhok – Marieke, Jula, Asiya, Rhea en Marghe - bedanken. Ladies, what would this PhD have been without you? For sure lower in calories and especially less fun. Thanks for all the laughs, cries and “kippendinners” we shared.

Tony, Arthur en Jurjen, bedankt voor alle technische hulpjes. Rien en Heidi, bedankt voor de elektronen microscopie. Tinca en Sophie, bedankt voor jullie adviezen over statistiek.

Chris, jij en de andere dierverzorgers weten de zorg voor de dieren en service voor de onderzoekers erg goed te combineren. Het was fijn om met jullie te werken.

Oliver, vielen Dank für deine Hilfe beim Tierversuch für Kapitel 3. Deine Effizienz, Pragmatismus und Professionalität sind mir ein großes Vorbild. Wer sonst plant für einen Versuch 3 Stunden und 20 Minuten und ist dann nach genau 3 Stunden und 17 Minuten fertig. Vielen Dank für die tolle Zusammenarbeit!

Lieve analisten, Desiree, Joost, Robbert, Ingrid, Joke, Frank, Bastijn, Boukje en Zabi, iedereen van jullie draagt met zijn eigen werk, techniek en ervaring zo ongelofelijk veel bij aan elk project in het lab. Bedankt voor alle muizen, cel kweek, constructen, virussen, genotyping en blotjes die mij ontzettend veel tijd en moeite hebben bespaard. Maar zeker nog belangrijker is de positieve atmosfeer die jullie in het lab creëren. Jullie zijn van onschatbare waarde in het lab!

Speciale dank gaat uit naar mijn paranimfen:
Marieke, patch-maatje, (hotel)kamergenootje en vriendin. Ik vond het super gezellig om al die jaren samen te studeren en te werken. Ik ben blij dat je mijn paranimf wilt zijn.

Jens, was als Stempelkurs für Autapsen in Göttingen anfing ist zu Freundschaft geworden. Dank dir für alle patch clamp Ratschläge, Tanzstunden, dein offenes Ohr und dass du jetzt sogar für mich sogar einen Frack anziehst.

Verder wil ik graag de leescomissie (Prof.dr. Sabine Spijker, Prof.dr. Casper Hoogenraad, Prof.dr. Gerard Borst en Prof.dr. Peter Burbach) bedanken voor de tijd de zij in mijn boekje hebben gestoken. Special thanks also to Dr. Johannes Hjorth for proofreading the manuscripts.

Mijn studenten en student assistenten Cillian, Susanne, Bart en Sophia. Bedankt voor jullie inzet en interesse in mijn project. Ik heb veel geleerd van jullie en heb er alle vertrouwen in dat jullie succesvolle wetenschappers zullen worden.

Next I want to thank my collaborators:
Casper, Samantha and Josta, I enjoyed working together on the Liprin-α2 story. It is nice to see it all fit together in the end. In Germany we have the saying “Aller guten Dinge sind 3!”, so lets keep fingers crossed for the next submission!

Johannes, what started as a Friday afternoon idea over a cup of tea, turned into my first publication and a tool that I am very proud of and used extensively for this thesis. Besides I learned a lot from this collaboration, not only scientifically. Keep up that curiousity! Science needs people like you!

Verder wil ik graag de andere afdelingen, INF en MCN, binnen het CNCR bedanken. Bij jullie stonden de deuren altijd voor mij open. Bedankt voor alle goede adviezen en hulp bij mijn nieuwe ideeën. Veel hiervan hebben tot de beste experimenten in dit boek geleid. In het bijzonder wil ik Tim, Jaap, Sabine, Priyanka, Rolinka, Patricia and Jörg bedanken. En niet te vergeten natuurlijk: Super-Hans! Bedankt voor de vele uurtjes patch-hulp en troubleshooting.

Life is more than just science! Bedankt iedereen die voor de nodige afleiding, ontspanning en vermaak heeft gezorgd:

Boukje, Irene en Marieke, ik heb al zin in ons volgend etentje.

Fabio, it has been a while since our last dance. But you’ll always be „the partner“, partner!

Sophia, bedankt voor alle kleurrijke naai-avonturen en -uitjes.

Nina, danke für die schönen Kaffeepausen an der VU.

En natuurlijk de vissersvrienden Michel, Desiree, Dick, Marie, Guido, Yoka, Tycho, Rianne, Daniel and Liane voor alle gezelligheid.

Atie, Henk, Robert, Meta en de kids, ook jullie wil ik bedanken voor jullie interesse in mijn proefschrift en de afleiding en steun als het even iets minder ging.

Mama, Anja, Stefan, Lukas, Philipp, Padi und Godi, danke für euer Interesse und Unterstuetzung, auch wenn es schwierig zu erklären ist was ich in den letzten 4 Jahren gemacht habe. Egal wie wild meine Pläne auch waren, auf euch konnte ich immer zählen. Daheim bleibt immer ein warmes Nest. Schön, dass es euch gibt!

Marcel, zonder deze AIO plek was ik je waarschijnlijk nooit tegengekomen in de `De Toffe`. Onze relatie was het beste “experiment” dat ik ooit ben begonnen en ik heb nu al zin in de komende avonturen. Ik hou van je!

Sabine