Materials and methods

7.1 Animals

Munc18-1 deficient mice were generated as described previously [53]. Munc18-1 null mutant mice are stillborn and can be easily distinguished from wild-type or heterozygous littermates. E18 embryos were obtained by caesarian section of pregnant females from timed matings of heterozygous mice. Newborn P0-P1 pups from pregnant female Wistar rats were used for glia preparations. Animals were housed and bred according to institutional, Dutch and U.S. governmental guidelines.

7.2 Constructs

Single amino acid substitutions in Munc18-1 (1b splice variant unless indicated otherwise) were generated either by subcloning fragments in PCRblunt (Invitrogen) and then cloning them into pIRES2EGFP (Clontech) or by generating them using Quickchange (Stratagene). The following constructs were used: Munc18-1a-ires-EGFP and Munc18-1b-ires-EGFP (Chapter 2), Munc18-1-2A-CreEGFP, Munc18-1(Y473A)-2A-CreEGFP and Munc18-1(Y473D)-2A-CreEGFP (Chapter 3), Munc18-1, Munc18-1(L130K), Munc18-1(F115E) and Munc18-1(E59K) (Chapter 4). In chapter 5, we used previously published Munc18-1 mutants generated using random transposon insertion and a kind gift from Prof. Dr. Alan Morgan (for cloning details see [81]). Transposon insertions of 5 amino acids were located after site Gln203, Leu307, Glu379, Glu420 and Phe540.

All constructs were verified by sequencing and subcloned into pLenti vectors, and viral particles were produced as described [169]. Transduction efficiencies were assessed on HEK293T cells using a concentration range, and were taken into account when viruses were applied to neuronal cultures. For this purpose,
HEK293T cells were infected 1 day after plating with lentivirus in DMEM medium containing 10% FCS (Gibco) and 1% penicillin/streptomycin (Gibco). Cells were fixed when confluence was reached. Infected cells were counted in at least 10 fields of view (40x objective) based on EGFP expression (if included in the construct) or immunostaining of Munc18-1.

7.3 **Protein expression and purification for biochemistry**

The bacterial expression constructs for SNARE proteins, cysteine-free SNAP-25a (1-206), the soluble portion of Syntaxin 1a (1-262), the soluble portion of Synaptobrevin 2 (1-96) have been described before. Likewise, the single-cysteine SNARE protein variants used for labeling Syb2 Cys79 has been published [31]. Full-length Munc18-1 and the three mutants M18L130K, M18F115E and M18E59K were constructed by site-directed mutagenesis. All constructs were cloned into a pET28a vector and expressed in *E. coli*. Proteins and SNARE complexes assembled from purified monomers were purified by Ni²⁺-NTA affinity chromatography followed by ion-exchange chromatography essentially as described [31].

7.4 **Isothermal titration calorimetry**

Isothermal titration calorimetry (ITC) was performed on a VP-ITC instrument (GE Healthcare) at 25 °C. Samples were dialyzed against degassed PBS buffer (20 mM sodium phosphate, pH 7.4, 150 mM NaCl, 1 mM DTT). Titrations were carried out by 20 µl injections. The measured heat released upon binding was integrated and analyzed with Microcal Origin 7.0 using a single-site binding model, yielding the equilibrium association constant $K_0$, the enthalpy of binding $\Delta H$, and the stoichiometry $N$. Experimental data are shown in (Supplemental Fig. 4.1).

7.5 **Fluorescence spectroscopy**

Fluorescence measurements were carried out in a Fluorolog 3 spectrometer in T-configuration equipped for polarization (Horiba Scientific). Single cysteine variants were labeled with Texas Red C5 bromoacetamide or Oregon Green 488 iodoacetamide according to the manufacturer’s instructions (Invitrogen). All experiments were performed at 25 °C in 1 cm quartz cuvettes in PBS buffer. Fluorescence anisotropy, which is used to indicate the local flexibility of the labeled residue, and which increases upon complex formation and decreases upon dissociation, were measured essentially as described [31]. The G factor was calculated according to $G = I_{HV}/I_{HH}$, where I is the fluorescence intensity, and the first
7.6 Denatured IPs and western blots

HEK293T cells were cultured in DMEM medium containing 10% FCS (Gibco) and 1% penicillin/streptomycin (Gibco). HEK293T cells were plated at equal density into 6-well plates 1 day before transfection. Cells were transfected with calcium phosphate transfection at 80% confluence and were allowed to grow for approximately 36 hours before harvesting. For calcium transfection, 150 µl 250 mM CaCl$_2$ was added to 150 µl filter sterilized 2x HEBS solution (274 mM NaCl, 10 mM KCl, 1.4 mM Na$_2$HPO$_4$.2H$_2$O, 1 mM dextrose and 42 mM HEPES in H$_2$O (pH 7.05)) mixed with 3 µg DNA under constant shaking. Precipitate was allowed to settle for 20 minutes after which it was added to a single well on a 6-well plate. Medium was replaced the next morning. For determining protein levels or performing denatured immunoprecipitation (IP), cells were lysed on ice with Laemmli Sample Buffer (LSB) containing 2% SDS, 10% glycerol, 2% β-mercaptoethanol, 60 mM Tris (pH 6.8).

For denatured IPs, samples were boiled for 5 minutes and DNA was sheared with an insulin syringe. A small percentage was taken for total protein levels and stored at -20 °C while the rest of the sample was diluted 15x in PKB buffer (50 mM Tris pH7.5, 1% Triton-X100, 1.5 mM MgCl$_2$, 5.0 mM EDTA and 100 mM NaCl) supplemented with protease inhibitor mix (Sigmacast 100x stock) and 1 mM of the protein-tyrosine phosphatase inhibitor Pervanadate. IPs were performed overnight at 4 °C using polyclonal rabbit Munc18-1 antibody (3 µl, SySy) conjugated to protein A Agarose beads (Sigma). Beads were washed 5x with alternating low and high salt PKB buffer (high salt buffer contains double the amount of NaCl as normal ‘low salt’ PKB buffer). Proteins were eluted by boiling in 1x LSB and analyzed by Western blotting.

For western blots, samples were loaded onto 10% SDS-PAGE gels and ran at 25 mA per gel until satisfactory mass separation. Proteins were then transferred to PVDF or nitrocellulose (in case of phosphoblot) membrane at 350 mA for 1hr at 4 °C. Membranes were blocked in TBST (TBS (pH 7.4) containing 0.1% TWEEN
20) supplemented with 3% BSA (in case of phosphoblot) or 2% milk powder and 0.5% BSA to reduce unspecific antibody binding. Primary antibodies were applied overnight at 4 °C (anti-Munc18-1 (monoclonal mouse (Ms), 1:5000, BD Biosciences), anti-Phosphotyrosine (monoclonal Ms, 1:2000, clone 4G10), anti-Flag (monoclonal Ms, 1:5000, Sigma), anti-Actin (monoclonal Ms, 1:2500, Millipore)). After washing, alkaline phosphatase-conjugated secondary antibodies (1:10000, Dako) were applied for 1hr at 4 °C. Blots were washed again, incubated with ECF substrate (GE Healthcare) for 5 minutes and scanned on a Fuji-film FLA-5000 Reader. If needed, blots were stripped for 1hr with stripping buffer (0.1 M Glycine pH 2.5-3), blocked and re-used for immunostaining. Results were analyzed using the Gel Analyzer tool in ImageJ (NIH, Bethesda, MD).

7.7 Glia preparation

Cerebral cortex of newborn rat pups (postnatal day 0-1) is incubated for 1hr at 37 °C in 5ml defrosted DMEM supplemented with 0.2 mg/ml L-Cysteine (free base), 1 mM CaCl₂, 0.5 mM EDTA and freshly added papain (20-25 units/ml). The supplemented DMEM solution has been bubbled with carbogen (5% CO₂ in O₂) for 10-20 minutes and filtered before use. The medium is then replaced with defrosted and filtered DMEM supplemented with 10% FCS/FBS, 2.5 mg/ml Albumin (bovine, fraction V) and 2.5 mg/ml Trypsin-Inhibitor (type II-O from chicken egg white). After 15 minutes incubation at 37 °C, the medium is replaced with glia medium (DMEM + glutamax (Invitrogen) supplemented with 10% FCS, 1%NEAA (100x stock, Sigma) and 1% Pen/Strep (100x stock, Sigma)). Cell are triturated with fire polished Pasteur pipette and plated in T175 flasks containing glia medium (one flask per rat). Medium was refreshed the next day and cells will reach confluence in one week.

Fresh glia were used for island cultures. To reduce microglia in continental cultures, glia were frozen and stored in liquid nitrogen. After thawing, glia were allowed to recover and divide for 1 week. Flasks containing glia were shaken overnight and washed with pre-warmed PBS. Glia were dissociated in trypsin (0.05% Trypsin - EDTA, Invitrogen) for 3-5 minutes at 37 °C and triturated in 10ml glia medium. Medium was then added to appropriate volume for plating in 12-well plates. After 4-5 days, medium is replaced by supplemented Neurobasal (see section below).

7.8 Dissociated neuronal cultures

Hippocampi and cortices were separately collected in ice-cold Hanks Buffered Salt Solution (HBSS; Sigma) buffered with 7mM HEPES (Invitrogen). After removal of the meninges, neurons were incubated in Hanks-HEPES containing 0.25% trypsin (from 10x stock, Invitrogen) for 20 minutes at 37 °C. After washing, neu-
rons were triturated using a fire-polished Pasteur pipette and counted in a Fuchs-
Rosenthal chamber. Neurons were plated in pre-warmed Neurobasal medium sup-
plemented with 2% B-27, 1.8% HEPES, 0.25% glutamax and 0.1% Pen/Strep (all
Invitrogen) onto various surfaces to create autaptic, network or glia-free cultures.
Munc18-1 null neurons are infected with lentiviral particles encoding Munc18-1
constructs several hours after plating, with the exception of network cultures in
which case neurons are infected prior to plating (see below).

To achieve autaptic cultures, hippocampal munc18-1 null neurons were plated
on micro-islands of rat glia at a density of 6K per well in a 12-well plate, and
infected with lentiviral particles several hours after plating. To generate these
micro-islands, glass coverslips (Menzel) were etched in 1M HCl for at least two
hours and neutralized with 1M NaOH for maximum one hour, washed thoroughly
with MilliQ water and washed once with 70% ethanol. Coverslips were stored in
96% ethanol and coated with agarose type II-A (0.0015% in H2O, Sigma) prior
to microdot application. Coating was done by spreading a thin layer of agarose
solution (heated in microwave and kept at 55 °C during use) with a cotton swab
over the entire coverslip. Microdots were created using a custom made rubber
stamp (dot diameter 250 µm) to apply solution consisting of 0.1 mg/ml poly-D-
lysine (Sigma), 0.7 mg/ml rat tail collagen (BD Biosciences) and 10 mM acetic
acid (Sigma) by stamping from a wet filter paper (3 mm cellulose chromatog-
raphy paper (Whatman)). Coverslips were UV-sterilized for 20 minutes before
further use. Astrocytes were plated at 6-8 K/well in pre-warmed DMEM medium
(Invitrogen) supplemented with 10% FCS, 1% nonessential amino acids and 1%
penicillin/streptomycin (all Gibco).

Network cultures were generated by plating cortical neurons (100-500 K per
well on a 12 well plate) on a confluent layer of rat glia grown on etched glass
coverslips (see procedure above) sprayed with the same solution used to make
the microdots for autaptic cultures. These neurons are infected prior to plating by
incubating the titrated neurons for 2 hours at 37 °C in complemented Neurobasal
medium containing lentiviral particles. 5 mM Ara-C (cytosine arabinoside) was
added to network cultures at DIV2 to inhibit DNA replication in order to reduce
microglia.

For protein level measurements, glia-free cultures were used. Neurons were
plated at 50 or 100 K/well in a 12-well plate containing glass coverslips that
were disinfected with 96% ethanol and subsequently coated with 0.5 milli-percent
poly-L-ornithine (Sigma) and 2 µg/ml laminin (Sigma) in PBS overnight and thor-
roughly washed.

### 7.9 Electrophysiological recordings

Autaptic or network cultures of munc18-1 null neurons were grown for 14-18 days
before measuring. Whole-cell voltage-clamp recordings (Vm = -70 mV) were per-
formed on DIV 13-18 at room temperature with borosilicate glass pipettes (2.5-
4.5 mOhm) filled with 125 mM K\(^+\)-gluconic acid, 10 mM NaCl, 4.6 mM MgCl\(_2\), 15 mM creatine phosphate, 10 U/ml phosphocreatine kinase and 1 mM EGTA (pH 7.30). External solution contained the following (in mM): 10 HEPES, 10 Glucose, 140 NaCl, 2.4 KCl, 4 MgCl\(_2\) and 4 CaCl\(_2\) (pH = 7.30, 300 mOsmol). In chapter 4, 20 \(\mu\)M gabazine (Sigma) was added to the external solution to isolate excitatory currents. In all other chapters, inhibitory neurons were identified and excluded based on the decay of postsynaptic currents. Recording were acquired with an Axopatch 200A amplifier (Molecular Devices), Digidata 1322A and Clampex 9.0 software (Molecular Devices). After whole cell mode was established, only cells with an access resistance of <12 M\(\Omega\) and leak current of <500 pA were accepted for analysis.

In autaptic neurons, EPSCs were elicited by a 0.5 ms depolarization to 30 mV. In chapters 2, 4 and 5, responses below 300 pA were excluded from analysis due to insufficient rescue. In chapter 2, more cells were excluded among neurons rescued with M18\(_{E59K}\) compared to other groups (M18\(_{WT}\), 24%; M18\(_{L130K}\), 29%; M18\(_{F115E}\), 28%; M18\(_{E59K}\), 48% excluded). In chapter 3, cells were not excluded based on EPSC size since a severe reduction in EPSC size was part of the phenotype. The RRP size was assessed either by the application of hypertonic sucrose (500 mM) or by giving an RRP depleting stimulation train (100 pulses at 40 Hz) [210, 222]. For the stimulation train, RRP size is estimated by back-extrapolation of the cumulative synchronous charge from the last 40 stimuli. The Y-axis intercept is used as a measurement of RRP size.

In network cultures, local field stimulation (1mA, 1ms) was applied with a bipolar concentric electrode placed in the vicinity of the patched neuron. Spontaneous release in networks was measured in the presence of 1 \(\mu\)M tetrodotoxin (Ascent) to block Na\(^+\) currents. Offline analysis of electrophysiology was performed using Clampfit v9.0 (Axon Instruments), Mini Analysis Program v6.0 (synaptosoft) and custom-written software routines in Matlab 7.1 or R2009b (Mathworks).

### 7.10 Immunocytochemistry

Neurons were allowed to develop for 13-18 days (glia containing cultures) or 10–11 days (glia-free cultures) before fixation. Cultures were fixed with 3.7% formaldehyde (Electron Microscopy Sciences). After washing with PBS, cells were permeated with 0.1% Triton X-100 for 5 minutes and incubated in 2% normal goat serum for 20 minutes to block nonspecific binding. Cells were incubated for 1 hr at room temperature in a primary antibody mixture of monoclonal mouse anti-VAMP (1:1000, SySy), polyclonal chicken anti-MAP2 (1:10000, Abcam) and polyclonal rabbit anti-Munc18-1(b) (1:500, SySy) antibodies. An anti-Munc18-1 antibody directed against site S241 (Phosphosolutions, polyclonal rabbit, 1:1000) was used to recognize both Munc18-1 splice variants in chapter 2. After washing, cells were incubated for 1 hr at room temperature with secondary antibodies con-
jugated to Alexa dyes (1:1000, Molecular Probes) and washed again. Coverslips were mounted with DABCO-Mowiol (Invitrogen) and images were acquired with a confocal microscope (LSM 510, Carl Zeiss) using a 40x oil immersion objective (NA = 1.3) with 0.7x zoom at 1024 x 1024 pixels and averaged over two scans. Confocal settings were kept the same for all scans within an experiment. Neuronal morphology and protein levels were analyzed using a published automated image analysis routine [220].

7.11 Electron Microscopy

Autaptic hippocampal cultures of munc18-1 null mutant mice (E18) obtained from 2 different litters were fixed at DIV14-16 for 45 min at room temperature with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) [54, 278]. As for electrophysiology, 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 hr at room temperature with 1% Osmium tetroxide/1% Potassium ferrocyanide in bidest, washed and stained with 1% uranyl acetate for 40 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. 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 with Image J (National Institute of Health, USA) on digital images of synapses taken at 100.000 x magnification using analySIS software (Soft Imaging System, Gmbh, Germany). 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. To get an estimate size of the total synaptic vesicle pool, distances of undocked synaptic vesicles to the active zone membrane were also included in our measurements.
7.12 Data analysis

Data are presented as mean values ± s.e.m., with n referring to the number of cells from each group unless stated otherwise. Statistical analysis was performed with 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 Bartlett. If data allowed an unpaired t-test (with Welch correction if standard deviations are not equal) or an ANOVA was used to determine statistical significance. Alternatively, the non-parametric Mann-Whitney U test or the Kruskal-Wallis Test (for comparing one or multiple groups, respectively) was used. If a test for multiple groups reached significance, post testing to compare the experimental to the control group was performed using the parametric Bonferroni post test or the nonparametric Dunn’s post test. P-values below 0.05 are considered significant and are indicated as following: *P<0.05, ** P<0.01, *** P<0.001.