Early Golgi abnormalities and neurodegeneration upon loss of presynaptic proteins Munc18-1, syntaxin-1 or SNAP-25

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Abstract

The loss of presynaptic proteins Munc18-1, syntaxin-1 or SNAP-25 is known to produce cell death, but the underlying features have not been compared experimentally. Here, we investigated these features in cultured mouse CNS and dorsal root ganglion neurons. Side-by-side comparisons confirmed massive cell death, before synaptogenesis, within 1-4 days in vitro (DIV) upon loss of t-SNAREs (syntaxin-1, SNAP-25) or Munc18-1, but not v-SNAREs (synaptobrevins/VAMP1/2/3 using Tetanus Neurotoxin (TeNT), also in TI-VAMP/VAMP7 knockout (KO) neurons). A condensed cis-Golgi was the first abnormality observed upon Munc18-1 or SNAP-25 loss within 3 DIV. This phenotype was distinct from the Golgi fragmentation observed in apoptosis. Cell death was too rapid after syntaxin-1 loss to study Golgi abnormalities. Syntaxin-1 and Munc18-1 depend on each other for normal cellular levels. We observed that endogenous syntaxin-1 accumulates at the Golgi of Munc18-1 KO neurons. However, expression of a non-neuronal Munc18 isoform that does not bind syntaxin-1, Munc18-3, in Munc18-1 KO neurons prevented cell death and restored normal cis-Golgi morphology, but not synaptic transmission or syntaxin-1 targeting. Finally, we observed that dorsal root ganglion neurons are the only Munc18-1 KO neurons that do not degenerate in vivo or in vitro. In these neurons, cis-Golgi abnormalities were less severe, with no changes in Golgi shape. Together these data demonstrate that cell death upon Munc18-1, syntaxin-1 or SNAP-25 loss occurs via a degenerative pathway unrelated to the known synapse function of these proteins and involving early cis-Golgi abnormalities, distinct from apoptosis.
Introduction

Neuronal survival is supported by a complex interplay of multiple molecular pathways\textsuperscript{34,224}. Loss of synapse integrity and function has received considerable attention as a putative starting point for cell loss and is considered an important early feature in many neurodegenerative disorders\textsuperscript{15,115,225,226}. However, how loss of synapse integrity/function leads to cell death is unclear.

One approach to study such links between synapses and cell loss has been to exploit genetic or proteolytic depletion of synaptic proteins in mouse models and \textit{in vitro}. Depletion of specific presynaptic proteins involved in exocytosis, Munc18-1, syntaxin-1 and SNAP-25, produces neuronal cell death\textsuperscript{25-28,155,157,219,227,228}. Neurodegeneration has also been observed for presynaptic proteins involved in endocytosis\textsuperscript{152,153}. However, depletion of other presynaptic proteins equally important for exocytosis, Munc13-1/2 or synaptobrevin-2/VAMP2, does not produce cell death\textsuperscript{30,31,221}, nor does depletion of postsynaptic proteins. Hence, the relationship between loss of (pre-)synaptic function and neuronal viability is complex and remains poorly understood.

It is conceivable that the role of these (pre-)synaptic proteins in neuronal viability is distinct from their role in synaptic transmission. In the first study (of Munc18-1 knockout (KO) neurons\textsuperscript{27}), it was proposed that neurons die due to defective synaptic transmission. However, in subsequent studies, on Munc13-1/2 double knockout (DKO) and synaptobrevin-2/VAMP2 KO neurons\textsuperscript{30,31,221}, synaptic transmission was also abolished but without cell death. Alternatively, it has been emphasized that some presynaptic proteins depend on each other for normal cellular levels. For instance, syntaxin-1 levels are 70% reduced in Munc18-1 KO neurons\textsuperscript{181,189} and Munc18-1 levels are reduced in syntaxin-1 KO\textsuperscript{175} and knock-in mice\textsuperscript{212}. In addition, Munc18-1 regulates targeting of overexpressed syntaxin-1 to the plasma membrane in heterologous cells\textsuperscript{191,193}, suggesting that syntaxin-1 and Munc18-1 are co-chaperones\textsuperscript{189-191,229}. Such interdependencies may contribute to cell viability. However, this interdependency is unlikely to explain cell loss in SNAP-25 KO neurons. Instead, cell loss in SNAP-25 KO neurons has been attributed to impaired release of trophic factors see\textsuperscript{28}, because high-density SNAP-25 KO cultures showed no degeneration\textsuperscript{215}. In contrast, even in intact brain, cell specific deletion of Munc18-1 expression in a subpopulation of neurons (i.e., within the normal network of trophic factor secreting neurons), produces loss of that population of neurons\textsuperscript{26}. Hence, different degenerative pathways may operate in these different models, probably unrelated to the shared role these proteins have in synaptic transmission.

To systematically compare the cell death mechanism(s) activated by depletion of presynaptic proteins, we performed a side-by-side study of Munc18-1 KO neurons, SNAP-25 KO neurons, Munc18-1/SNAP-25 DKO neurons and syntaxin-1 depletion using \textit{Botulinum Neurotoxin C (BoNT/C) versus} several non-degenerative models, synaptobrevin/VAMP depletion using \textit{Tetanus Neurotoxin (TeNT), TI-VAMP/VAMP7 KO neurons and Munc13-1/2 DKO neurons. To monitor and compare cell loss with high cellular/time resolution, we used cultured primary mouse neurons and dorsal root ganglia (DRG) explants and monitored viability, protein targeting and synaptic transmission. We found that cell death upon Munc18-1,
syntaxin-1 or SNAP-25 depletion occurs via a degenerative pathway unrelated to the known synaptic function of these proteins and identified early cis-Golgi abnormalities, distinct from apoptosis, as the most early sign of neurodegeneration.

Material and Methods

Animals
Munc18-1 KO, SNAP-25 KO, Munc13-1/2 DKO and TI-VAMP KO mice were generated as described previously. Munc18-1/SNAP-25 DKO mice were generated by crossing Munc18-1 HZ with SNAP-25 HZ. Embryonic day 18 (E18) or E14 embryos were obtained by caesarian section of pregnant females from timed mating. Animals were housed and bred according to the Institutional, Dutch and US governmental guidelines.

Neuronal cultures
Hippocampi and cortices were separately collected in ice-cold Hanks Buffered Salt Solution (HBSS; Sigma) buffered with 7 mM HEPES (Invitrogen). Meninges were removed and neurons incubated in Hanks-HEPES with 0.25% trypsin (Invitrogen) for 20 min at 37°C. After washing, neurons were triturated with fire polished Pasteur pipettes 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). Hippocampal neurons were plated at a density of 6K/well on 18 mm glass coverslips on micro islands of rat glia. Glial islands were created by plating 8 K/well rat glia on UV-sterilized agarose-coated etched glass coverslips stamped with a 0.1 mg/ml poly-d-lysine (Sigma) and 0.2 mg/ml rat tail collagen (BD Biosciences) solution. Network cultures were created by plating 25-50 K/well on 18 mm glass coverslips or 250K/well on a 35 mm glass bottom dish of cortical neurons on a confluent layer of rat glia grown on etched glass coverslips sprayed with a 0.1 mg/ml poly-d-lysine and 0.2 mg/ml rat tail collagen (BD Biosciences) solution.

Dorsal Root Ganglia Cultures
Dorsal Root Ganglia (DRG) from E14 Munc18-1 KO mice of were dissected (~ 45 DRGs per animal) in ice-cold Hanks Buffered Salt Solution (HBSS; Sigma) buffered with 1 M HEPES (Invitrogen). Washed DRGs were transferred to coated coverslips with Poly-L-lysine 0.1 mg/ml (Sigma) and Matrigel (BD). DRGs were placed in the middle of the coverslip on top of a drop of Neurobasal (Invitrogen) supplemented with 45% D-(-)-glucose (Sigma), 1% Glutamax (Gibco), 2% B-27 (Invitrogen) and 50 ng/ml Nerve Growth Factor (Sigma).

Constructs, Lentiviruses and chemicals
Neuronal cortical cultures were infected at DIV 0 with lentiviral particles encoding SNAP-25 (S25), Munc18-1 WT (M18-1), Munc18-3 (M18-3), Tetanus Neurotoxin (TeNT), Botulinum Neurotoxin Type C (BoNT/C) expressing EGFP, mCherry or no tagged fluorescent protein for DIV 3 and DIV 14 experiments, together with control lentiviruses expressing enhanced
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green fluorescent protein (EGFP). Secretory vesicle marker neuropeptide Y (NPY) fused to mCherry was described before and delivered using lentiviral particles at DIV 0. For live cell imaging, cultures were infected with RFP as a morphological marker and ManII-GFP (gift from V. Malhotra, Centre for Genomic Regulation, Barcelona, Spain) as a cis-Golgi marker, which was delivered with lentiviral particles at DIV 0. F-actin was visualized live using mCherry-UtrCH (gift from William Bement (Addgene plasmid # 26740), which was delivered using lentiviral particles at DIV 0. For electrophysiology recordings, hippocampal cultures were infected with Munc18-1 WT, Munc18-2 and Munc18-3 cDNAs linked to EGFP via an internal ribosomal entry site (IRES).

In order to induce apoptosis, neuronal cultures were treated with 1 μM Staurosporine for 3h or DMSO as control.

**Immunocytochemistry and confocal microscopy**

Cortical neuronal cultures were fixed at DIV 3, 5, 7, 9, 11 and 14 and DRG cultures at DIV 1, 6, 13 and 21 with 4% formaldehyde (Electron Microscopy Sciences). After washing with Phosphate Buffered Saline (PBS), neurons were permeabilized with 0.5% Triton X-100, followed by a 30 min incubation in PBS containing 0.1% Triton X-100 and 2% normal goat serum to block aspecific binding. The same solution was used for diluting antibodies. Neurons were then stained with primary antibodies for 2h at room temperature (RT), washed with PBS, and stained with Alexa Fluor-conjugated secondary antibodies (1:1000; Invitrogen) for 1h at RT. Phalloidin rhodamine (1:1000; ThermoFisher Scientific) probe was stained together with the secondary antibodies. The primary antibodies and dilutions used were chicken anti-MAP2 (1:10000; Abcam), mouse anti-GM130 (1:1000; BD Transduction), rabbit anti-syntaxin-1 (1:1000; clone I379, gift from T. Südhof, Department of Molecular and Cellular Physiology and Howard Hughes Medical Institute, Stanford University School of Medicine, CA, USA), rat anti-LAMP1 (1:100; Abcam), rabbit anti-calnexin (1:500; ENZO Life Sciences), mouse anti-Human Transferrin Receptor (1:500; Invitrogen), chicken anti-Neurofilament (1:2000; Novus Biologicals), mouse anti-VAMP (1:100; clone 69.1, SySy), guinea pig anti-vGlut (1:10000; Millipore), mouse anti-SMI312 (1:1000; BioLegend), and anti-tubulin (1:500; SySy). After an additional 3 washes, coverslips were mounted on microscopic slides with Mowiol-DABCO and imaged on a NIKON Ti-Eclipse microscope, with a confocal scanner model A1R+. Image acquisition was performed using the resonant scanning mode, using a 60X oil immersion objective (NA 1.40) with 2x zoom. Z stacks were acquired with 1 μm interval for apoptotic markers. For cell survival, a 40X oil immersion objective (NA 1.30) was used to scan an area with a radius of 1.5mm (97 fields of view); or on a Zeiss 510 Meta Confocal microscope (Carl Zeiss) with a 63X Plan-Neofluar lens (NA 1.4, Carl Zeiss b.v. Weesp) with or without 4x zoom, z stacks were acquired with 0.63 μm interval, or 10X lens (NA 0.3, Carl Zeiss b.v. Weesp); Neuronal organelles morphology and co-localization was analyzed using ImageJ software, z stacks images were collapsed to maximal projection and analyzed. Total dendrite length, total number of presynaptic terminals and dendrite branching (using Sholl analysis) were measured using custom written software routines in Matlab. For Golgi fragmentation analyses, Matlab
software was used and a stack of several slices was loaded in the interface and converted to a 3D matrix of size w x h x z and thresholded using the Otsu method. The resulting binary image stack was further processed to examine the connectedness of the voxels in a 26-neighborhood fashion. The resulting objects were quantified by calculating the surface and volume of each object, while the number of objects was used as a measure for fragmentation.

**Live cell imaging**

For live mitochondria experiments, cortical neuronal cultures were incubated, at DIV 3, with 50 mM of MitoTracker Green FM (Life Technologies, gift from N. Raimundo, Institute for Cellular Biochemistry, University Medical Center Göttingen, Germany) for 20 min at 37°C with 5% CO₂. Afterwards coverslips were placed in an imaging chamber with Tyrode’s solution (2 mM CaCl₂, 2.5 mM KCl, 119 mM NaCl, 2 mM MgCl₂, 20 mM glucose and 25 mM HEPES, pH 7.4) and imaged live at RT on a Zeiss 510 Meta Confocal microscope (Carl Zeiss) with a 63X Plan-Neofluar lens (NA 1.40, Carl Zeiss b.v. Weesp). For live Golgi and UtrCH experiments, cortical neurons were plated on a 35 mm glass bottom dish, 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). Neurons were relocated at DIV 3 to a NIKON Ti-Eclipse microscope, with a confocal scanner model A1R+, and a Tokai Hit heating system with heated stage, water bath and objective, pre-warmed to 37°C and regulated CO₂ to 5%. For Golgi experiment images were acquired every minute using the resonant scanning mode for 2h15min, with a 60X oil immersion objective (NA 1.4) and 2x zoom, z stacks were acquired with 1 µm interval. For live UtrCH images were acquired using the resonant scanning mode, with a 60X oil immersion objective (NA 1.4), z stacks were acquired with 1 µm interval.

**Brain lysate and Western blot**

Brain lysate for western blot analysis was homogenized in ice-cold PBS with protease inhibitors (PI) (Sigma) and then incubated for 2h at 4°C in 1% Triton X-100 in 1x PBS with PI. The brain lysate was then centrifuged for 20 min at 10,000 g at 4°C. 1 ml Laemmli Sample Buffer (LSB) was added per 0.1 g of brain and boiled for 20 min at 100°C. Samples were loaded on 15% SDS-PAGE gel. Acrylamide gels were transferred onto 0.2 µm pore size Immune-Blot PVDF membrane for protein blotting (Bio-Rad Laboratories). Membranes were incubated on an orbital shaker for 30 min at RT in 2% Skim milk powder (Merck), 0.5% Bovine Serum Albumin (BSA) (Thermo Fisher Scientific) containing 0.1% Tween-20 (TBS-T) (Sigma) to block non-specific binding. Primary antibody incubation was done in 1% PBS-Tween or overnight at 4°C, followed by 3 washes with PBS-T. Membranes were stained with secondary antibody conjugated with alkaline phosphatase (AP) or Alexa-647 (1:1000) (Jackson ImmunoResearch) for 1h at RT. After 3 washes with TBS-T the AP conjugated antibody was visualized using an attophos AP fluorescent substrate (Promega). Membranes were scanned with a Fuji Image FLA-5000 Reader and analyzed with ImageJ software. Primary antibodies used: anti-TI-V AMP (1:1000; SySy), anti-Munc18 (1:1000, clone # 610336, BD Transduction Laboratories), anti-SNAP-25 (1:1000, SMI-81, Sternberger) and anti-γ-Tubulin (1:2000, clone # GTU-88, Sigma).
Electrophysiological Recordings
Whole cell voltage-clamp (Vm = -70 mV) recordings of Munc18-1 KO neurons rescued with Munc18-1-ires-EGFP or Munc18-3-ires-EGFP were performed on DIV 10-15. The patch pipette solution contained (in mM): 125 K⁺-gluconic acid, 10 NaCl, 4.6 MgCl₂, 4 K2-ATP, 15 Creatine Phosphate, 1 EGTA and 20 U/ml Phosphocreatine Kinase (pH 7.30). The external medium used contained the following components (in mM): 140 NaCl, 2.4 KCl, 4 CaCl₂, 4 MgCl₂, 10 HEPES, 10 Glucose (pH 7.30). We used fast double barrel application of extracellular (500 mM) hypertonic sucrose to reliably assess RRP size. Axopatch 200A was used for whole-cell recordings. All recordings were at 33°C. Signal was acquired using Digidata 1322A and Clampex 8.1. Clampfit 8.0 was used for offline analysis.

RNA isolation, cDNA synthesis and real-time qPCR
Cortical neuronal cultures, DRG neurons and brain tissue were lysed and scraped in TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and organic and aqueous phase separation was initiated by the addition of and mixing with chloroform (Merck, Whitehouse Station, NJ, USA). Subsequently, RNA isolation was performed automated with an RNeasy MiniKit on a Qiacube (Qiagen, Venlo, the Netherlands; according to the manufacturer’s protocol). RNA purity and integrity was assessed spectrophotometrically on a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, MA, USA). cDNA synthesis was performed on 200 ng of RNA per reaction (RNA sample quantities do not vary within each experiment) using a SuperScript II Reverse Transcriptase Kit (Invitrogen). Priming of mRNA poly-A tails was performed with 125 pmol oligo(dT)₁₂-VN primer in a final volume of 10 μL and was incubated at 70°C for 10 min. MgCl₂ (2 mM), dNTPs (0.5 mM), 5 μL 5 × First-Strand Buffer and 100 U SuperScript II reverse transcriptase were added to the primed mRNAs in a final volume of 25 μL per reaction and incubated at 42°C for 60 min. The reverse transcription reaction was stopped by incubation at 70°C for 10 min. Per sample 1 μL cDNA was pipetted in triplicate into a 384-well plate and dried in a DNA110 SpeedVac (Thermo Scientific). Primers (forward and reverse) used for Munc18-1 5’-AACATTGCCTTTCTCCCCTA-3’, 5’-GAAAGAGTCAGCGGAGTCCA-3’; Munc18-2 5’-GCGGCACTGTCACCAACT-3’, 5’-CAGCGAGACAGCTGGTAGG-3’; Munc18-3 5’-TCTCGATGGACACCTTTTATCA-3’, 5’-CGGCCACTCTTTGGAATCTA-3’; mEEFA1 (Mouse Eukaryotic Elongation Factor 1A) 5’-ACACGTAGATTCCGGCAAGT-3’, 5’-AGGAGCCCTTTCCCATCTC-3’. Roche Universal Probes used Munc18-1 #95, Munc18-2 #19, Munc18-3 #88, mEEFA1 #31.

Results
Rapid neuronal loss upon depletion of syntaxin-1/2/3, Munc18-1 or SNAP-25, but not synaptobrevin/VAMP1/2/3 and/or VAMP7/TI-VAMP
To systematically study how depletion of different presynaptic proteins relates to cell death, we monitored neuronal survival in parallel cortical cultures of Munc18-1 KO, SNAP-25 KO, Munc18-1/SNAP-25 DKO, WT, WT neurons infected with lentiviral vectors expressing TeNT which cleaves synaptobrevin/VAMP1/2/3 proteins and BoNT/C which
cleaves syntaxin-1/-2/-3 and SNAP-25\textsuperscript{204,235}. BoNT/C dependent cleavage of SNAP-25 is less efficient\textsuperscript{204,220,236-238} and not relevant for neuronal viability\textsuperscript{221}. Therefore we consider BoNT/C expression as a tool to study syntaxin-1/-2/-3 dependent viability. All cultures were grown on rat (wild-type) glia feeders to provide optimal support for neuronal viability independent of the genetic background of the neurons. TeNT and BoNT/C-expressing viral vectors used a synapsin-1 promoter, which does not induce expression in glia feeders (data not shown). Cultures were fixed at different time points (DIV 3, 5, 7, 9, 11 and 14) and immunocytochemistry was performed using MAP2, to count neurons and study their morphology. MAP2 positive cells were counted in 97 fields of view for each experimental condition. WT neurons all survived in culture during the first 5 days. A fraction of these neurons died during the second week, 39.8\% at DIV 14 (Fig. 2.1A, B). Similarly, WT neurons treated with TeNT showed no significant cell death during the first week in culture and survival was comparable to WT neurons in the second week (Fig. 2.1A, B). However, WT neurons treated with BoNT/C died before DIV 3, while 45.8\% of Munc18-1 KO neurons and 51.2\% SNAP-25 KO neurons survived at DIV 3. For Munc18-1/SNAP-25 DKO only 17.1\% of neurons survived by DIV 3 and appeared underdeveloped. Beyond DIV 3 further cell loss was observed for Munc18-1 KO, SNAP-25 KO and Munc18-1/SNAP-25 DKO neurons (Fig. 2.1A, B). Only in the case of SNAP-25 KO a fraction of the neurons survived and by DIV 14, 4.1\% of normally developed neurons were alive (Fig. 2.1A, B). Taken together, these data show that considerable heterogeneity exists in neuronal viability of cultured neurons lacking specific presynaptic proteins.

The fact that depletion of the v-SNAREs (synaptobrevins) produces such a different effect on cell survival than depletion of t-SNAREs (syntaxins and SNAP-25) or Munc18-1 might be explained by the expression of Synaptobrevin7/TI-VAMP, which is resistant to TeNT\textsuperscript{239}. TI-VAMP is expressed at normal levels in both Munc18-1 and SNAP-25 KO neurons (Fig. 2.1C). However, TeNT expression in neuronal cortical cultures of TI-VAMP KO mice showed no significant increase in cell loss up to DIV 14 compared to control (TI-VAMP WT) neurons (92.7\% TI-VAMP WT treated with TeNT and 99.4\% for TI-VAMP KO treated with TeNT; ns p > 0.05 using one-way ANOVA followed by the post hoc Bonferroni’s test; Fig. 2.1D-E). Together, these data show that depletion of v-SNAREs or t-SNAREs/Munc18-1 has very different effects on neuronal viability. The fact that cell death occurs before synaptogenesis, points to a distinct role of t-SNAREs/Munc18-1, but not v-SNAREs in neuronal survival. Furthermore, Munc18-1/SNAP-25 DKO neurons show more severe cell loss than the individual mutants (additivity), suggesting that the underlying degenerative pathways are not the same for SNAP-25 and Munc18-1 depletion.

**Abnormal cis-Golgi morphology is an early phenotype in Munc18-1 and SNAP-25 KO neurons**

To investigate subcellular compartments involved in neuronal survival/death, we studied the Golgi morphology using cis-Golgi markers GM130 and Mannosidase II; the size and number of mitochondria using MitoTracker; the ER compartment using anti-calnexin antibody; lysosomes using anti-LAMP1 antibody; recycling endosomes using anti-transferrin receptor...
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Figure 2.1 - Neuronal loss upon depletion of t-SNAREs and Munc18-1 but not v-SNAREs. A, Cortical neuronal cultures from Wild-type (WT), WT infected with TeNT or BoNT/C, Munc18-1 knockout (M18-1 KO), SNAP-25 (S25) KO and Munc18-1/SNAP-25 DKO (M18/S25 DKO) were fixed at different time points (DIV 3, 5, 7, 9, 11 and 14) and stained with a dendritic marker (MAP2). B, Quantification of number of neurons per 97 fields of view; ns p > 0.05; *** p < 0.001 using two-way ANOVA followed by the post hoc Bonferroni’s test. C, E18 brain lysates from WT, M18-1 KO and S25 KO were analyzed by Western blot and showed no differences in TI-VAMP levels between mutants and control animals (n=3). D, Cortical neuronal cultures from TI-VAMP WT and TI-VAMP KO infected with TeNT or RFP (as control) were fixed at DIV 14 and stained with a dendritic marker (MAP2). E, Quantification of number of neurons per field of view. No differences in cell survival were found ns p > 0.05 using one-way ANOVA followed by the post hoc Bonferroni’s test; shown are mean ± SEM.
the mutant neurons (Fig. 2.2A, B). The size of the soma was also smaller in the mutant neurons, but this difference was minor relative to the changes in Golgi morphology and remained constant between DIV 2-3 (Fig. 2.2C). The differences in Golgi size were still significant after correcting for the difference in soma size (by quantifying cis-Golgi extent as a fraction of the soma, Fig. 2.2D). cis-Golgi structure was further analyzed using automated shape analysis (the ImageJ ‘round’ parameter). This analysis confirmed the condensed Munc18-1 KO cis-Golgi when compared to the elongated WT cis-Golgi structure (Fig. 2.2E). Mannosidase II (ManII), a Golgi enzyme, was used as a cis-Golgi live marker and independent approach to confirm the effects observed using GM130 staining. ManII imaging indeed showed a significant decrease in cis-Golgi area when compared to WT (Fig. 2.2F, G).

Golgi abnormalities are also a prominent feature of apoptosis. Therefore, we tested whether the observed abnormalities in cis-Golgi morphology were similar to the abnormalities observed in apoptosis by applying 1μM of Staurosporine for 3h, which induces apoptosis. Using Matlab, a Golgi 3D structure was generated from confocal images and the number of Golgi fragments was counted. Upon apoptosis induction, a fragmented Golgi was observed in both WT and Munc18-1 KO neurons, with a significant increase in the fragment numbers (Fig. 2.2H, I). Hence, the changes observed in cis-Golgi morphology upon the induction of apoptosis were similar in both genotypes and distinct from the condensed cis-Golgi observed in Munc18-1 KO neurons. These two approaches, using GM130 staining and ManII imaging, indicate that Munc18-1 depletion leads to Golgi abnormalities, distinct from typical apoptotic fragmentation, before cells die.

No significant differences were observed in recycling endosomes, ER, or lysosomes between WT and Munc18-1 KO (Fig. 2.2J). A tendency towards bigger mitochondria was observed in WT compared to Munc18-1 KO, 1.3 μm² versus 1.1 μm², respectively (Fig. 2.2K, L), but the difference was not significant (p > 0.05 using Student’s t-test) and the suggested effect size (< 20%) was minor relative to the (significant) Golgi defects (50% difference, Fig. 2.2C-F). The distribution of mitochondria per neurite also showed no differences, (0.1 and 0.1 μm⁻¹ for WT and Munc18-1 KO, respectively; ns p > 0.05 using Student’s t-test; Fig. 2.2M). In addition, we tested the biogenesis and targeting of secretory vesicles in developing neurons. NPY-Cherry, a marker for secretory vesicle was expressed at the time of plating and the distribution of secretory vesicles was analyzed at DIV 3. In both control and Munc18-1 KO neurons, secretory vesicles accumulated at the tips of the outgrowing neuritis (Fig. 2.2N). This indicates that during early development, trafficking of secretory vesicles to the ends of outgrowing neurites is not affected by the loss of Munc18-1 and therefore it cannot explain cell death in these neurons. Hence, Munc18-1 KO neurons show a selective defect in cis-Golgi morphology, not in other organelles and distinct from Golgi abnormalities in apoptosis, within 2 days in culture, well before synapse formation.

To study if the condensed cis-Golgi is a specific feature of Munc18-1 KO cell death, neuronal cultures from other genotypes were also tested: SNAP-25 KO neurons, Munc13-1/2 DKO and WT neurons treated with TeNT. All these genotypes are characterized by severe defects in synaptic transmission, comparable to the Munc18-1 KO phenotype. WT
neurons treated with BoNT/C were not analyzed, because cell death occurred too rapidly after plating, before DIV 3 (Fig. 2.1A, B), to allow Golgi comparisons with the other genotypes. WT neurons treated with TeNT and Munc13-1/2 DKO neurons showed no Golgi abnormalities (Fig. 2.3A-E). In contrast, SNAP-25 KO neurons showed a significant defect in cis-Golgi shape (Fig. 2.3E), similar to the Munc18-1 KO neurons (Fig. 2.2E), and a trend towards a smaller size (not significant, Fig. 2.3B). SNAP-25 KO neurons and SNAP-25 KO neurons infected with GFP showed a trend towards a smaller cis-Golgi, as observed in Munc18-1 KO neurons: 14.0 μm², 13.7 μm² (ns p = 0.208 and p = 0.474, respectively, using one-way ANOVA followed by the post hoc Bonferroni's test) and a significantly smaller soma size in SNAP-25 KO neurons 125.5 μm², but not in SNAP-25 KO infected with GFP 132.8 μm², when compared to WT neurons (** p < 0.01, *** p < 0.001 using one-way ANOVA followed by the post hoc Bonferroni's test, Fig. 2.3A-C). While overexpression of SNAP-25 in SNAP-25 KO neurons restored the cis-Golgi shape defect observed in SNAP-25 KO neurons, overexpressing of Munc18-1 or -3 did not restore the cis-Golgi shape in SNAP-25 KO neurons (* p < 0.05, *** p < 0.001 using one-way ANOVA followed by the post hoc Bonferroni's test, Fig. 2.3A, E). The Munc18-1/SNAP-25 DKO mutant showed a smaller cis-Golgi and also a smaller cis-Golgi/soma ratio when compared to WT (Fig. 2.3A-D), and also when compared to the single KO’s. Thus, cis-Golgi abnormalities are an early characteristic of Munc18-1 KO and SNAP-25 KO neurons. The additive effect of Munc18-1 and SNAP-25 deficiency suggests that absence of these two presynaptic proteins leads to cell death via pathways that are not fully overlapping.

**Munc18-3 expression rescues survival and Golgi abnormalities, but not synaptic transmission and syntaxin-1 targeting defects in Munc18-1 KO neurons**

Overexpression of a SNAP-25 isoform, SNAP-23, rescues cell death of SNAP-25 KO neurons. To investigate if expression of a non-neuronal Munc18 isoform (Munc18-3) rescues survival of Munc18-1 KO neurons, Munc18-3 was overexpressed by lentiviral infection in Munc18-1 KO neurons and fixed at DIV 3, 7, 9 and 14 for immunocytochemistry. At DIV 3, Munc18-1 KO neurons rescued with Munc18-1 or Munc18-3 showed 100% and 90.9% viable cells, respectively, compared to 68.3% of Munc18-1 KO neurons expressing control virus (GFP only; Fig. 2.4A, B). At DIV 7 and 9, few Munc18-1 KO neurons remained, as shown in Fig. 2.1 (4.7% and 5.8%, surviving cells, respectively), while in Munc18-1 KO neurons rescued with Munc18-1, but also with non-neuronal Munc18-3 no reduction in cell count was observed (Fig. 2.4A-B). Only after two weeks in culture, Munc18-1 KO neurons rescued with Munc18-3 started to show cell loss (Fig. 2.4A, B). The number of synapses and the length of dendrites analyzed at DIV 14 showed no significant differences between neurons rescued with Munc18-1 or -3 (Fig. 2.4C-E). These data show that a non-neuronal Munc18 isoform rescues the viability and neuronal morphology of Munc18-1 KO neurons until DIV 14.

Staining for GM130 revealed that Munc18-3 expression in Munc18-1 KO neurons also restored cis-Golgi abnormalities (Fig. 2.4F-J). As before, cis-Golgi morphology was quantified as a total Golgi area (Fig. 2.4G), as fraction of the soma (Fig. 2.4G-I) and using the 'round' parameter (Fig. 2.4J). These analyses showed no differences in cis-Golgi morphology...
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Figure 2.2 – Cell loss in Munc18-1 KO neurons is preceded by abnormal cis-Golgi morphology, but no changes in other organelles. A, Cortical neurons from WT and Munc18-1 (M18-1) KO, fixed at DIV 2 and DIV 3 were stained for a cis-Golgi marker (GM130) and a dendritic marker (MAP2). B, cis-Golgi area is smaller in Munc18-1 KO. cis-Golgi (GM130) area was measured quantitatively using ImageJ software; DIV 2 WT: 14.3 μm² ± 1.13; Munc18-1 KO: 9.2 μm² ± 0.70; ** p < 0.01 using two-way ANOVA; DIV 3 WT: 24.0 μm² ± 1.26; Munc18-1 KO: 11.1 μm² ± 0.58; *** p < 0.001

Figure 2.2 and legend continue on next pages >>>
Golgi abnormalities upon loss of presynaptic proteins

H

K

L

M

J

N

WT

KO

Munc18-1 KO

DMSO

Staurosporine

Mito / μm neurites

WT

KO

Munc18-1 KO

TfR

Calnexin

MAP2

Lamp1

SMI312

MAP2

NPY-Cherry

merge

TfR

MAP2

Calnexin

MAP2

Lamp1

MAP2

SMI312

N of Golgi Fragments

* * *
between Munc18-1 and -3 expression in Munc18-1 KO neurons and significant differences to Munc18-1 KO neurons infected with GFP only (Fig. 2.4F-J).

Golgi integrity and dynamics are regulated by interactions between the Golgi and the cytoskeleton and actin depolymerization leads to a condensed Golgi similar to the Golgi abnormalities observed in Munc18-1 KO neurons. Therefore, we analyzed the main cytoskeletal components in neurons, tubulin and actin, in Munc18-1 KO and control neurons. Cortical cultures were fixed at DIV 3 and immunohistochemistry was performed for tubulin, phalloidin (a high affinity F-actin probe), neurofilament and MAP2. No difference in phalloidin intensity was observed between mutant and control neurons (1811.6 A.U. ± 106.88 and 1798.2 A.U. ± 153.67, WT and Munc18-1 KO, respectively; ns p > 0.05 using Student's t-test; Fig. 2.5A-C).

Furthermore, cortical cultures were infected with ManII-GFP a cis-Golgi marker and calponin homology domain of utrophin (UtrCH-Cherry) a probe to visualize F-actin and imaged live. No difference was observed between WT and Munc18-1 KO neurons in UtrCH intensity at the cis-Golgi (2575.4 A.U. ± 117.11 and 2457.0 A.U. ± 160.61, WT and Munc18-1 KO, respectively; ns p > 0.05 using Student's t-test; Fig. 2.5A-C).

Hence, both the tubulin and actin cytoskeleton had developed normally in Munc18-1 KO neurons, also specifically around the Golgi, and therefore we conclude that the early Golgi abnormalities cannot be explained by cytoskeletal defects.

Munc18-3 rescued synapse formation in Munc18-1 KO neurons (Fig. 2.4D), therefore we studied synaptic transmission in these cells using whole-cell voltage clamp...
Figure 2.3 – Depletion of SNAP-25 but not synaptobrevins or Munc13-1/2 DKO leads to changes in cis-Golgi morphology. A, Cortical neuronal cultures from WT, WT treated with TeNT, Munc13-1/2 DKO (M13 DKO), SNAP-25 KO +, Munc18-1, Munc18-3, M18/S25 DKO. B, GM130 Area (μm²) for WT +, TeNT, M13-1/2 DKO, S25 KO +, M18-1, M18-3, M18/S25 DKO. C, Soma Area (μm²) for WT +, TeNT, M13-1/2 DKO, S25 KO +, M18-1, M18-3, M18/S25 DKO. D, Golgi/Soma Ratio for WT +, TeNT, M13-1/2 DKO, S25 KO +, M18-1, M18-3, M18/S25 DKO. E, Round for WT +, TeNT, M13-1/2 DKO, S25 KO +, M18-1, M18-3, M18/S25 DKO. Legend continues on next page...
25 KO (S25 KO), SNAP-25 KO rescued with EGFP, SNAP-25, Munc18-1 or -3 and Munc18-1/SNAP-25 DKO (M18/S25 DKO), were fixed at DIV 3 and stained with a cis-Golgi marker (GM130) and a dendritic marker (MAP2). B, cis-Golgi (GM130) morphology was analyzed quantitatively using ImageJ software; WT: 17.2 μm² ± 0.75; WT with TeNT: 19.0 μm² ± 1.93; Munc13-1/2 DKO: 21.6 μm² ± 1.68; SNAP-25 KO: 14.0 μm² ± 0.65; SNAP-25 KO with GFP: 13.7 μm² ± 0.99; SNAP-25 KO with SNAP-25: 16.2 μm² ± 1.89; SNAP-25 KO with Munc18-1: 16.5 μm² ± 1.07; SNAP-25 KO with Munc18-3: 16.1 μm² ± 0.87; Munc18-1/SNAP-25 DKO: 2.4 μm² ± 0.27; *** p < 0.001 using one-way ANOVA followed by the post hoc Bonferroni's test.

C, Soma (MAP2) area was measure quantitatively using ImageJ software; WT: 170.7 μm² ± 6.16; WT with TeNT: 183.1 μm² ± 10.94; Munc13-1/2 DKO: 197.0 μm² ± 10.23; SNAP-25 KO: 125.5 μm² ± 4.70; SNAP-25 KO with GFP: 132.8 μm² ± 7.50; SNAP-25 KO with SNAP25: 139.6 μm² ± 10.97; SNAP-25 KO with Munc18-1: 149.8 μm² ± 7.50; SNAP-25 KO with Munc18-3: 162.0 μm² ± 7.74; Munc18-1/SNAP-25 DKO: 73.4 μm² ± 5.61; *** p < 0.001 using one-way ANOVA followed by the post hoc Bonferroni's test.

D, cis-Golgi area corrected for the soma area; WT: 1.0 ± 0.03; WT with TeNT: 1.0 ± 0.07; Munc13-1/2 DKO: 1.1 ± 0.06; SNAP-25 KO: 1.1 ± 0.04; SNAP-25 KO with GFP: 1.0 ± 0.05; SNAP-25 KO with SNAP-25: 1.1 ± 0.06; SNAP-25 KO with Munc18-1: 1.1 ± 0.06; SNAP-25 KO with Munc18-3: 1.0 ± 0.04; Munc18-1/SNAP-25 DKO: 0.3 ± 0.03; *** p < 0.001 using one-way ANOVA followed by the post hoc Bonferroni's test. E, cis-Golgi shape was measure quantitatively using ImageJ software; WT: 0.4 ± 0.03; WT with TeNT: 0.4 ± 0.04; Munc13-1/2 DKO: 0.4 ± 0.04; SNAP-25 KO: 0.6 ± 0.02; SNAP-25 KO with GFP: 0.6 ± 0.03; SNAP-25 KO with SNAP-25: 0.4 ± 0.04; SNAP-25 KO with Munc18-1: 0.5 ± 0.04; SNAP-25 KO with Munc18-3: 0.6 ± 0.04; Munc18-1/SNAP-25 DKO: 0.6 ± 0.05; * p < 0.05, *** p < 0.001 using one-way ANOVA followed by the post hoc Bonferroni's test; shown are mean ± SEM.

experiments. The amplitude and decay time of spontaneous release events (mEPSC) were comparable between neurons rescued with either Munc18-1, -2 or -3. However, Munc18-2 and -3 expressing neurons showed virtually no spontaneous release: the frequency was approximately 4% and 2% of the frequency observed in Munc18-1 expressing neurons (Fig. 2.6A, B). In addition, action potential driven evoked EPSC amplitudes were reduced in Munc18-2 expressing neurons and virtually absent in Munc18-3 expressing neurons, to 22% and 8% of Munc18-1 amplitude, respectively; while evoked EPSC decay time was normal (Fig. 2.6C, D). The decrease in miniature frequency and evoked EPSC amplitude can be caused by either a decrease in the pool of ready releasable vesicles (RRP) or a decrease in vesicular release probability (P_{ves}). We probed the RRP by application of hyperosmotic sucrose solution (500 mM) and found a large reduction in RRP size in both, Munc18-2 and -3 expressing neurons, to 3% and 1.5% of Munc18-1 RRP size, respectively (Fig. 2.6E, F). Evoked EPSC amplitude and RRP size were proportionally reduced in Munc18-2 and -3 expressing neurons. Therefore, the initial release probability (evoked EPSC charge/initial sucrose charge) of the few vesicles that were released upon stimulation appeared to be similar between Munc18-1 and Munc18-2 and -3 expressing neurons (Fig. 2.6E, F). Hence, while neuronal viability is rescued by expressing Munc18-2 and -3, synaptic transmission is not.

It has been reported before that targeting of syntaxin-1 to the plasma membrane is impaired in the absence of Munc18-1. As such defects might contribute to cis-Golgi abnormalities observed in Munc18-1 KO neurons, co-staining of syntaxin-1 and GM130 was performed. At DIV 3, Munc18-1 KO neurons showed a significant increase in co-localization of syntaxin-1 and GM130, when compared with WT neurons (0.08 and 0.45 Pearson's correlation, WT and Munc18-1 KO, respectively; *** p < 0.001 using one-way ANOVA followed by the post hoc Bonferroni's test; Fig. 2.7A, B). SNAP-25 KO neurons, although having Golgi abnormalities,
Figure 2.4 – Munc18-3 expression rescues cell death, neuronal and cis-Golgi morphology in Munc18-1 KO neurons. A, Cortical neuronal cultures from Munc18-1 KO were infected by lentiviral particles with EGFP (control), Munc18-1 (M18-1) or Munc18-3 (M18-3), fixed at different time points (DIV 3, 7, 9 and 14) and stained with a

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showed no significant differences in syntaxin-1 co-localization with cis-Golgi, when compared to WT neurons (0.14 Pearson’s correlation; ns p > 0.05 using one-way ANOVA followed by the post hoc Bonferroni’s test; Fig. 2.7A, B). At DIV 14, WT neurons and Munc18-1 KO neurons rescued with Munc18-1 or -2 showed a negative Pearson’s correlation between syntaxin-1 and GM130 (-0.358, -0.359 and -0.255 Pearson’s correlation, WT, Munc18-1 KO rescued with Munc18-1 and -2, respectively; ns p > 0.05 using one-way ANOVA followed by the post hoc Bonferroni’s test; Fig. 2.7C, D). Despite the fact that cis-Golgi morphology is normal in Munc18-1 KO neurons expressing Munc18-3, analysis of co-localization between syntaxin-1 and GM130 showed a positive Pearson’s correlation of 0.17, significantly different from WT and Munc18-1 KO neurons expressing Munc18-1 or -2 (*** p < 0.001 using one-way ANOVA followed by the post hoc Bonferroni’s test; Fig. 2.7C, D). The synaptic syntaxin-1 levels were also analyzed by co-staining Munc18-1 KO neurons expressing Munc18-1, -2 or -3 at DIV 14 with syntaxin-1 and synaptobrevin-2/VAMP2. No differences in synaptobrevin-2/VAMP2 intensity were observed (163.1 A.U. ± 7.0, 150.3 A.U. ± 5.8 and 169.0 A.U. ± 6.1 Munc18-1 KO rescued with Munc18-1, -2 and -3, respectively; ns p > 0.05 using one-way ANOVA followed by the post hoc Bonferroni’s test) (Fig. 2.7C, F). A significant decrease in the synaptic syntaxin-1 staining was observed in Munc18-1 KO neurons expressing Munc18-2 or -3 when compared to Munc18-1 (ratio syntaxin-1/synaptobrevin-2 1.12 ± 0.04, 0.91 ± 0.07 and 0.87 ± 0.05 Munc18-1 KO expressing Munc18-1, -2 and -3, respectively; * p < 0.05 using one-way ANOVA followed by the post hoc Bonferroni’s test; Fig. 2.7G). Together, these data show that while viability, neuronal morphology and Golgi abnormalities are rescued by expressing Munc18-3 and Munc18-2, syntaxin-1 targeting is not rescued by Munc18-3, while Munc18-2 produces an intermediate phenotype, suggesting that syntaxin-1 targeting defects are not causal to cell death observed upon Munc18-1 loss.
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Figure 2.5 – Munc18-1 KO neurons show no cytoskeleton organization defects. A, Cortical neuronal cultures of WT and Munc18-1 KO were fixed at DIV 3 and stained with neurofilament and tubulin antibodies and phalloidin (a F-actin probe); merge image: tubulin – green, phalloidin – red, neurofilament - blue. B, Cortical neuronal cultures of WT and Munc18-1 KO were fixed at DIV 3 and stained with MAP2 and phalloidin. C, Quantification of somatic phalloidin intensity was performed using ImageJ software. No differences were observed between mutant and control neurons; WT: 1811.6 A.U. ± 106.88; Munc18-1 KO 1798.2 A.U. ± 153.67; ns p > 0.05 using Student’s t-test. D, WT and Munc18-1 KO cortical neurons expressing UtrCH-Cherry, (a live probe to visualize F-actin), and ManII-GFP (a live cis-Golgi marker) were image at DIV 3. E, Example of region-of-interest (ROI) placement. F, Quantification of UtrCH intensity was performed at the cis-Golgi and the area around labeled as ring ((Intensity ROI 2 x Mean ROI 2) – (Intensity ROI 1 x Mean ROI 1))/(Area ROI 2 – Area ROI 1) using ImageJ software; ROI 1 WT: 2575.4 A.U. ± 117.11, ROI 1 Munc18-1 KO: 2457.0 A.U. ± 160.61; ns p > 0.05 using Student’s t-test; Ring WT: 1442.3 A.U. ± 127.54, Ring Munc18-1 KO: 1606.0 A.U. ± 177.04; ns p > 0.05 using Student’s t-test. G, No differences in actin and Golgi co-localization in Munc18-1 KO neurons; WT: 0.69 ± 0.03; Munc18-1 KO: 0.58 ± 0.04; measured quantitatively using ImageJ software; ns p > 0.05 using Student’s t-test; shown are mean ± SEM.
Figure 2.6 – Munc18-3 expression does not rescue synaptic transmission in Munc18-1 KO neurons. A, Example traces of spontaneous release in neurons rescued with Munc18-1 (M18-1), Munc18-2 (M18-2) or Munc18-3 (M18-3). B, Spontaneous frequency is impaired in neurons expressing Munc18-2 or -3, while spontaneous amplitude and decay time are normal; amplitude: Munc18-1 26.9 ± 1.9 pA; Munc18-2 22.0 ± 1.8 pA; Munc18-3 23.0 ± 4.5 pA; ns p > 0.05; frequency: Munc18-1 23.5 ± 0.2 Hz; Munc18-2 1.0 ± 0.2 Hz; Munc18-3 0.5 ± 0.3 Hz; *** p < 0.0001, decay time: Munc18-1 2.8 ± 0.2 msec; Munc18-2 2.6 ± 0.2 msec; Munc18-3 2.7 ± 0.7 msec; ns p > 0.05). C, Examples of evoked EPSCs in neurons expressing Munc18-1, -2 and -3. D, EPSC amplitude is significantly reduced in neurons expressing non-neuronal isoforms of Munc18, while EPSC decay time is normal (EPSC amplitude: Munc18-1 2441.5 ± 304.8 pA; Munc18-2 548.8 ± 167.9 pA; Munc18-3 188.3 ± 39.7 pA; ns p > 0.05, EPSC decay time: Munc18-1 3.4 ± 0.3 msec; Munc18-2 2.2 ± 0.3 msec; Munc18-3 2.8 ± 0.6 msec; ns p > 0.05). E, Ready releasable pool size, probed by hyperosmotic sucrose application, is severely decreased in neurons expressing Munc18-2 or -3 (Munc18-1 0.72 ± 0.08 nC; Munc18-2 0.02 ± 0.003 nC; Munc18-3 0.01 ± 0.005 nC; ** p < 0.01). F, Release probability of fusion competent vesicles is normal in neurons expressing Munc18-2 and -3 (Munc18-1 4.5% ± 0.7; Munc18-2 5.6% ± 0.4; Munc18-3 5.1% ± 0.9; ns p > 0.05); shown are mean ± SEM.

Munc18-1 KO DRG neurons survive in culture without compensatory Munc18-2 or -3 expression
In contrast to Munc18-1 KO neurons in the CNS and most notably in the spinal cord, the sensory neurons of the dorsal root ganglion (DRG) of Munc18-1 KO mice do not show signs of degeneration until birth. To investigate if Munc18-1 KO DRG neurons are also distinct from other neurons with respect to the cellular abnormalities observed in this study, DRG neurons were isolated from E14 animals, cultured as whole explants, fixed at different time...
Golgi abnormalities upon loss of presynaptic proteins

points (DIV 1, 6, 13 and 21) and stained using anti-neurofilament antibody. We found that Munc18-1 KO DRG neurons survived in culture as long as WT DRG neurons, until DIV 21 (Fig. 2.8A). For comparison, we also analyzed cortical neurons obtained at the same age as the DRGs (E14 instead of E18 as before, Fig. 2.1). In agreement with the data obtained from E18 cultures, the E14 Munc18-1 KO CNS neurons also died, albeit slightly slower: at DIV 5 approximately 50% of Munc18-1 KO neurons were still viable, followed by rapid deterioration leaving less than 8% of viable cells at DIV 7 (Fig. 2.8B-C). Hence, Munc18-1 KO CNS neurons cultured at an earlier developmental stage (E14) also deteriorate within a week, but in cultured DRG neurons Munc18-1 is not crucial for survival.

Survival in DRG neurons might be explained by (compensatory) expression of other Munc18 isoforms that does not occur in other neurons. Therefore, mRNA levels of Munc18-1, -2 and -3 were quantified, relative to a mEEF1A control (Mouse Eukaryotic Elongation Factor 1A), in cerebral cortex and DRG neurons of WT and Munc18-1 KO animals. No differences in the relative levels of the three Munc18 isoforms (normalized to mEEF1A) were observed between cerebral cortex and DRG neurons (Fig. 2.8D, E). Thus, the distinctive viability of Munc18-1 KO DRG neurons cannot be explained by expression of Munc18 isoforms and/or by compensatory changes in their expression profile in the absence of Munc18-1.

Munc18-1 KO DRG neurons have a similar syntaxin-1 targeting phenotype as CNS neurons
Munc18-1 KO DRG neurons were further analyzed for similar cellular changes as in CNS neurons using immunohistochemistry for a cis-Golgi marker, syntaxin-1 and neurofilament. The cis-Golgi size was significantly smaller in Munc18-1 KO DRG neurons (Fig. 2.9A, B), as well as the soma size (Fig. 2.9C), similar to Munc18-1 KO CNS neurons (Fig. 2.2A-E). When normalized to soma area, the decrease in Munc18-1 KO DRG Golgi was still significant (Fig. 2.9D). However, the Golgi shape (roundness) was not different (Fig. 2.9E). Syntaxin-1 mislocalization was apparent in Munc18-1 KO DRG neurons, as a positive Pearson’s correlation was found between syntaxin-1 and GM130 in Munc18-1 KO DRG neurons, significantly different from WT DRG neurons (-0.12 and 0.20, WT and Munc18-1 KO, respectively; *** p < 0.001 using Mann-Whitney U test; Fig. 2.9F). Thus, syntaxin-1 targeting defects and reduction in cis-Golgi size, but not shape, were also evident in DRG neurons, although these neurons do not die until DIV 21.

Discussion
In this study we showed that under the same conditions, t-SNAREs and Munc18-1 are crucial for neuronal survival, but v-SNAREs are not. A condensed cis-Golgi was observed as an early feature of neuronal cell death. Defects in synaptic transmission and, in the case of cell death in Munc18-1 KO, syntaxin-1 targeting defects were excluded as causal to cell death.

Similar roles for t-SNAREs and Munc18-1 but not v-SNAREs in neuronal viability
We observed massive cell loss in the absence of presynaptic proteins Munc18-1, and t-SNAREs (SNAP-25 or syntaxins) in cultured neurons (Fig. 2.1A,B), but not upon loss of all major
**Figure 2.**

**A** DIV 3 GM130, Syntaxin-1, MAP2, and Co-localization images for Wild-type, SNAP-25 KO, and Munc18-1 KO conditions.

**B** Pearson's Correlation (DIV 3) for GM130/Syntaxin-1.

**C** DIV 14 GM130, Syntaxin-1, MAP2, and merge images for Wild-type, Munc18-1 KO, Munc18-1 KO + Munc18-2, and Munc18-1 KO + Munc18-3 conditions.

**D** Pearson's Correlation (DIV 14) for GM130/Syntaxin-1.

**E** DIV 14 Syntaxin-1 and VAMP merge images for Munc18-1 KO + Munc18-2, Munc18-1 KO + Munc18-3, and Munc18-3 conditions.

**F** VAMP Intensity (A.U.) for Munc18-1 KO + Munc18-2, Munc18-1 KO + Munc18-3, and Munc18-3 conditions.

**G** Synaptic Syntaxin levels (Syntaxin/VAMP) for Munc18-1 KO + Munc18-2, Munc18-1 KO + Munc18-3, and Munc18-3 conditions.
neuronal v-SNAREs (treating TI-VAMP KO neurons with tetanus toxin; Fig. 2.1D, E). These data are consistent with previous studies showing that loss of t-SNAREs and Munc18-1 lead to neuronal cell death and that synaptobrevin-2/VAMP2 KO neurons and WT neurons treated with TeNT do not degenerate. Hence, t-SNAREs and the SM-protein Munc18-1, but not v-SNAREs have a crucial function in neuronal viability. Although t-SNAREs and Munc18-1 appear to have similar roles in neuronal viability, their impact is not identical. Under the same conditions created in this study, cell loss proceeded at a different pace in the absence of the different proteins. Syntaxin proteolysis led to the fastest cell loss, within 3 DIV, as shown before, while in SNAP-25 KO cultures a fraction of neurons survived beyond the first week, as shown before (Fig. 2.1A, B). We have now shown that Munc18-1/SNAP-25 DKO leads to cell death earlier than the two single KOs (Fig. 2.1A, B). This indicates that the survival pathway(s) supported by t-SNAREs and Munc18-1 depends on these proteins to a different extent during different phases of neuronal development. It seems plausible that functionally related proteins operate in the same pathway(s) until a given developmental phase and that the timing of expression of such proteins determines the exact time point where survival becomes dependent on t-SNAREs and Munc18-1.

Loss of synaptic transmission is not causally related to cell loss
In all conditions studied here, in vitro cell loss in the absence of t-SNAREs or Munc18-1 occurred well before synapse formation. This confirms previous findings on Munc18-1 KO and syntaxin KO neurons and indicates that the role of these proteins in neuronal survival is unrelated to their synaptic function. Furthermore, in contrast to cell loss observed in neurons exposed to BoNT/C, neurons exposed to BoNT/A, which selectively cleaves SNAP-25, show no signs of cell death, while synaptic transmission is blocked and mEPSCs are abolished. The current study provides a different kind of evidence for this conclusion: Munc18-2 and -3 expression in Munc18-1 KO neurons did not rescue synaptic transmission defects (Fig. 2.6), as expected for Munc18-3 since it does not bind syntaxin-1 in its closed conformation, but does rescue viability. Hence, loss of synaptic transmission is not causally related to the loss of neuronal viability. In addition to their established role in synaptic transmission, t-SNAREs and Munc18-1 have a separate, earlier role in neuronal viability.
Syntaxin-1 targeting defects are not causal to cell loss in Munc18-1 KO neurons

Neurons lacking Munc18-1 showed syntaxin-1 mislocalization at the Golgi (Fig. 2.7, 2.9), suggesting that without Munc18-1, syntaxin-1 export from the Golgi apparatus to the PM

**Figure 2.8 – Munc18-1 KO DRG neurons survive in culture without compensatory expression of Munc18-2 or -3.**

A, DRG cultures form E14 animals of WT and Munc18-1 (M18-1) KO were fixed at different time points (DIV 1, 6, 13 and 21) and stained with Neurofilament antibody; no degeneration was observed in Munc18-1 KO DRG neurons. B, Cortical neuronal cultures form E14 animals, WT and Munc18-1 KO, were fixed at different time points and stained for a dendritic marker (MAP2), at DIV 7 Munc18-1 KO neurons were few and underdeveloped. C, Quantification of number of neurons per 97 fields of view showed a decrease in percentage of surviving Munc18-1 KO cells, but not WT cells. D, mRNA levels of Munc18-1, -2 and -3 were quantified in DRG neurons from E14 animals, no changes were observed; Munc18-1 WT: 1.0 A.U. ± 0.19; KO: 0.0 A.U. ± 0.00; Munc18-2 WT: 1.0 A.U. ± 0.12; KO: 1.0 A.U. ± 0.06; Munc18-3 WT: 1.0 A.U. ± 0.07; KO: 0.84 A.U. ± 0.13; ns p > 0.05, * p < 0.05 using Mann-Whitney U test; shown are mean ± SEM. E, mRNA levels of Munc18-1, -2 and -3 were quantified in the cerebral cortex from E18 animals, no changes were observed; Munc18-1 WT: 1.0 A.U. ± 0.00; KO: 0.0 A.U. ± 0.00; Munc18-2 WT: 1.0 A.U. ± 0.00; KO 1.1 A.U. ± 0.04; Munc18-3 WT: 1.0 A.U. ± 0.00; KO: 1.05 A.U. ± 0.21; ns p > 0.05, * p < 0.05 using Mann-Whitney U test.
and synapses is impaired. This is consistent with previous studies in non-neuronal cells where syntaxin-1 overexpression alone produces Golgi abnormalities, but if transfected together with Munc18-1 localizes to the PM and no Golgi abnormalities were observed. A similar mislocalization of syntaxin was observed in the current study upon expression of Munc18-3 in Munc18-1 KO neurons. However, in these neurons the cis-Golgi abnormalities and cell death were rescued (Fig. 2.7C, D). Similarly, in Munc18-1 KO DRG neurons syntaxin-1 targeting was also impaired, but no cell death was observed (Fig. 2.8, 2.9). Taken together, these data indicate that syntaxin-1 targeting defects are not causal to the Golgi abnormalities or cell

Figure 2.9 – Munc18-1 KO DRG neurons are underdeveloped, show a smaller cis-Golgi and syntaxin-1 target defects. A, DRG cultures from E14 animals, WT and Munc18-1 (M18-1) KO, were fixed at DIV 21 and stained for syntaxin-1, cis-Golgi (GM130) and Neurofilament. B, Munc18-1 KO DRG neurons show a smaller cis-Golgi area WT: 52.0 μm² ± 6.20; Munc18-1 KO: 18.2 μm² ± 2.35; *** p < 0.001 using Mann-Whitney U test. C, Munc18-1 KO DRG neurons show a smaller soma area WT: 318.9 μm² ± 20.24; Munc18-1 KO: 159.1 μm² ± 9.26; *** p < 0.001 using Mann-Whitney U test. D, cis-Golgi area corrected for soma area shows that Munc18-1 KO cells have a reduced cis-Golgi, WT: 1.0 ± 0.08; Munc18-1 KO: 0.7 ± 0.07; ** p < 0.01 using Mann-Whitney U test. E, cis-Golgi shape shows no difference in cis-Golgi morphology; WT: 0.8 ± 0.02; Munc18-1 KO: 0.8 ± 0.02; ns p > 0.05 using Mann-Whitney U test. F, Munc18-1 KO DRG neurons show a Pearson's correlation coefficient between syntaxin-1 and GM130 of 0.20 ± 0.03 compared to Pearson's correlation coefficient in WT DRG neurons -0.12 ± 0.03; *** p < 0.001 using Mann-Whitney U test; shown are mean ± SEM.
death in Munc18-1 KO neurons.

Trophic factor secretion defects cannot explain cell loss upon t-SNARE/Munc18 depletion

It has been proposed that Munc18-1 and SNAP-25, in addition to synaptic transmission, are involved in trophic factor release, from neurons or glia, and this may provide an explanation for the cell loss in the absence of t-SNAREs or Munc18-1\textsuperscript{28,157,215}. Consistent with this idea, SNAP-25 neurons survived at least 3 weeks when cultured at very high density\textsuperscript{215} and syntaxin-1B KO neurons survived on WT, but not KO glial feeders\textsuperscript{157}. In the current study, we used selective BoNT/C (light chain) expression in neurons, not glia, while previous studies used the holotoxin or syntaxin-1 inactivation, which do not discriminate between neurons and glia. With selective BoNT/C expression in neurons only, cell loss is still massive (Fig. 1)\textsuperscript{29}. Furthermore, upon sparse munc18-1 inactivation only in Purkinje cells of mouse cerebellum, loss of these neurons was still massive, despite the presence of surrounding neurons and glia providing trophic support\textsuperscript{26}. Taken together, these considerations suggest that inhibition of trophic factor release is insufficient to explain the cell loss upon depletion of at least syntaxin and Munc18-1. Instead, early Golgi abnormalities and the fact that viability can be rescued by expression of non-synaptic isoforms (see below) suggest that cell intrinsic trafficking defects are the main cause of neurodegeneration upon t-SNARE or Munc18-1 depletion.

Condensed cis-Golgi morphology is an early hallmark of neurodegeneration

While defects in synaptic transmission and syntaxin-1 targeting can be excluded as causal for degeneration, and lack of trophic factor secretion appears to be an insufficient explanation, the identity of the degenerative mechanism(s) remains unclear. Previous studies have reported neurite fragmentation followed by cytochrome C release from mitochondria and apoptosis after BoNT/C holotoxin application\textsuperscript{220,221}. The current study shows that depletion of Munc18-1, SNAP-25 or both produced cis-Golgi abnormalities at an early development stage (Fig. 2.2, 2.3), while no differences in ER, lysosomes, recycling endosomes or mitochondria were observed (Fig. 2.2K-M). Conversely, in neurons that do not degenerate, e.g. Munc13-1/2 and synaptobrevin/VAMP loss, no Golgi defects were observed. While the cis-Golgi abnormalities were not identical upon depletion of Munc18-1, SNAP-25 or both, together they are a selective, early feature of neurodegeneration in all these models. This feature was distinct from the fragmented cis-Golgi observed in apoptosis (Fig. 2.2)\textsuperscript{39,135,136}, suggesting this phenomenon is upstream of the apoptosis observed in BoNT/C treated neurons\textsuperscript{220,221}. The exact nature of the abnormal Golgi remains unclear. One plausible scenario is that dysregulation of intracellular trafficking (fusion/fission reactions) leads to a net reduction in Golgi membrane and therefore a smaller size, but other scenarios cannot be excluded until further studies have been performed, especially at the ultrastructural level in Munc18-1 and SNAP-25 KO neurons. Interestingly, Golgi defects are also an early pre-clinical feature that precedes neurodegenerative loss of cell bodies and axons in Alzheimer’s disease\textsuperscript{147,150}, Amyotrophic Lateral Sclerosis\textsuperscript{140,143} and Parkinson’s disease\textsuperscript{151}. Thus, our data suggest Golgi defects as an early, shared hallmark preceding cell death upon depletion of t-SNAREs or Munc18-1.
**Rescue of viability by non-synaptic isoforms of t-SNAREs/Munc18 suggests generic membrane trafficking defects**

Expression of Munc18-2 and -3 in Munc18-1 KO neurons rescued neuronal viability (Fig. 2.4, 2.7). Similarly, expression of SNAP-23 rescues viability of SNAP-25 KO neurons and syntaxin-2, -3 and -4 rescue viability of syntaxin-1 depleted neurons. Hence, rescue of viability by non-cognate isoforms is a shared characteristic among the presynaptic proteins that cause neuronal loss upon their depletion. It seems plausible that these isoforms compensate in generic membrane trafficking pathways in neurons, required for neuronal maintenance and are more tolerant to differences among isoforms (Munc18-1 or -2/-3; SNAP-23 or -25, syntaxin-1 or -2/-4) than in the case of synaptic transmission. Selective defects in cis-Golgi were observed in at least two of the cases (Munc18-1 and SNAP-25 depletion) while in the last case (syntaxin depletion), cell loss was too rapid to detect Golgi defects reliably. This places these generic membrane trafficking pathways most upstream in the regulated secretory pathway, probably ER to cis-Golgi or a retrograde pathway to the Golgi.