Munc18-1 is involved in homeostatic upregulation of transmitter release at the myasthenic mouse neuromuscular synapse

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Abstract

Homeostatic upregulation of presynaptic neurotransmitter release in response to a reduction of postsynaptic transmitter sensitivity has been observed at brain synapses and neuromuscular junctions (NMJs). This phenomenon allows synapses to adapt to (patho-)physiological changes. Compensatory upregulation of ACh release has been observed at NMJs of patients with myasthenia gravis and in experimental animal models for this paralytic auto-immune disease, in which postsynaptic ACh receptors at NMJs are reduced by autoantibodies. This form of synaptic plasticity presumably involves local retrograde signaling from post- to presynapse. Ultimate targets of such signaling pathway may be the protein components of the neuro-exocytotic machinery or proteins that modulate its function. One candidate is Munc18-1, a presynaptic protein which is essential for neurotransmitter release and presumably acts as positive regulator of transmitter release at mouse NMJs. Munc18-1 is known to interact with the protein complex that regulates membrane fusion of transmitter vesicles. Here, we have investigated Munc18-1 involvement in homeostatic up-regulation of transmitter release at NMJs. To this end, we induced an experimental form of myasthenia gravis to Munc18-1 heterozygous null-mutant mice, Munc18-1 overexpressing transgenic mice, and wild type controls. Neurotransmitter release was measured with in vitro electrophysiological methods. Myasthenic Munc18-1 heterozygous null-mutant mice were impaired in their ability to upregulate acetylcholine release at their NMJs. On the other hand, Munc18-1 overexpressing NMJs did not show additional upregulation, compared to wild-type controls. We conclude that Munc18-1 is involved in homeostatic upregulation of transmitter release, and that other factors become limiting when Munc18-1 is present at elevated level.

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Introduction

Successful synaptic transmission between a neuron and its target cell relies on mutual tuning of pre- and postsynaptic structure and function. This is of particular importance during embryonic development when the physical properties of cells, e.g. cell input resistance, change drastically as a result of their growth. Throughout later stages in life there are also further (patho-)physiological changes that require structural and/or functional synaptic adaptation to ensure appropriate signal transmission. The molecular mechanisms by which neuronal cells monitor and adapt their synaptic strength are not well understood yet (Davis and Bezprozvanny, 2001; Davis, 2006; for review, see Turrigiano et al., 1998; Regehr et al., 2009).

One form of synaptic adaptation is the increase of presynaptic neurotransmitter release following a reduction in postsynaptic sensitivity for the transmitter. It has been suggested that a retrograde messenger is involved, signaling from the postsynaptic cell to the presynaptic cell. Although synaptic adaptation has been observed at CNS synapses (Bacci et al., 2001), this apparent homeostatic adaptive change has been best characterized at vertebrate and invertebrate neuromuscular junctions (NMJs). For example, increase of glutamate release from motor nerve terminals has been observed at the Drosophila NMJ, either upon lowering the number of postsynaptic glutamate receptors or inhibiting their function with transgenetic methods (Davis et al., 1998; Petersen et al., 1997). In transgenic mice with reduced expression of neuregulin and a consequently impairment of acetylcholine receptor (AChR) synthesis, the reduction of functional postsynaptic AChRs leads to compensatory increase in presynaptic ACh release at the NMJ (Sandrock, Jr. et al., 1997).

Similarly, compensatory increase of ACh release has been found at NMJs of patients suffering from myasthenia gravis (MG), in which autoantibodies against the AChR cause a reduction of receptor density resulting in muscle weakness (Vincent et al., 2001). In vitro electrophysiological analysis of NMJ function in muscle biopsies from MG patients revealed (partial) compensatory upregulation of ACh release (Cull-Candy et al., 1980; Plomp et al., 1995). MG has been studied using experimental models in rat, where the AChR function has been decreased by the use of antibodies or by chronic in vivo application of the AChR blocker α-bungarotoxin. In these models, a similar upregulation of ACh release was demonstrated (Plomp et al., 1992; Plomp et al., 1995). Detailed electrophysiological studies showed that the amount of this upregulation at each individual (experimental) myasthenic NMJ was depending on the amount of reduction of postsynaptic AChRs at that NMJ (Plomp et al., 1992; Plomp et al., 1995). This strongly suggests that local postsynaptic factors are signaling in a retrograde fashion to the presynaptic cell, as also suggested for Drosophila NMJs (Aberle et al., 2002; Davis et al., 1998; McCabe et al., 2003;
Petersen et al., 1997), cricket CNS synapses (Davis and Murphey, 1993) and rat hippocampal synapses (Micheva et al., 2003).

The molecular mechanism underlying retrograde signaling in the homeostatic control of ACh release at myasthenic NMJs is largely unknown. Retrograde signaling is accomplished either by diffusible retrograde messenger or through a transsynaptic protein complex (Regehr et al., 2009). Neurotrophic factors are candidates for diffusible messengers, since in vitro pharmacological block of the tyrosine kinases receptors reduces the increased ACh release levels at experimental MG NMJs to normal values (Plomp and Molenaar, 1996). Furthermore, nitric oxide has been shown to act as retrograde messenger in hippocampal synapses (Micheva et al., 2003). Interesting candidates for forming a transsynaptic signalling protein complex are e.g. neureligins and neurexins (Futai et al., 2007; Scheiffele, 2003).

The ultimate target of the retrograde signaling cascade leading to increased release is likely to be the presynaptic exocytotic machinery, dedicated to fast and precisely timed fusion of synaptic vesicles with the plasma membrane. A vast number of proteins is involved in exocytosis (see for reviews (Rosenmund et al., 2003; Sudhof, 2004; Verhage and Toonen, 2007; Toonen and Verhage, 2007), but the key event in synaptic vesicle fusion is the formation of the so-called SNARE complex (Poirier et al., 1998; Sutton et al., 1998). This is a parallel four-helix bundle formed by the SNARE motifs of the three neuronal SNARE proteins (syntaxin-1 and SNAP25 on the plasma membrane, and vesicle membrane-associated synaptobrevin). The energy released by the formation of this complex catalyzes the fusion reaction that leads to transmitter release (Finley et al., 2002; Hanson et al., 1997; Lin and Scheller, 1997).

The protein family Sec-Munc18 is necessary for exocytosis in yeast (Aalto et al., 1992; Novick et al., 1981), C. elegans (Hosono et al., 1992), Drosophila (Harrison et al., 1994) and mice (Verhage et al., 2000) and was initially discovered as a binding to syntaxin (Hata et al., 1993). Munc18 can either bind to the to the ‘closed’ form of syntaxin, or to syntaxin participating in the SNARE complex. In the ‘closed’ form of syntaxin, the N-terminus folds back onto the Habc domain forming a four helical structure which prevents participation of the protein in the SNARE complex (Dulubova et al., 1999; Misura et al., 2000). The arch-like structure of Munc18 binds as a clasp to the four helical syntaxin structure. The other mode of binding involves association of the N-terminus of Munc18 with the N-terminus of syntaxin while assembled in the SNARE complex, leaving the arch-shape structure of Munc18 free to clasp the four helices of the SNARE complex (Dulubova et al., 2007; Shen et al., 2007; Yamaguchi et al., 2002). How exactly Munc18 is involved in secretion is not clear yet, but most likely Munc18 is a catalyst for SNARE complex formation, which in turn drives membrane fusion (Sudhof and Rothman, 2009; see for reviews Toonen and Verhage, 2007).
Based on the fact that Munc18-1 is a key player in exocytosis, the protein could be involved in the release-increasing retrograde signaling cascade at myasthenic NMJs. In a previous paper, we have described the effect of varying the levels of Munc18-1 on transmitter release in mice by the use of heterozygous Munc18-1 null-mutant mice (Munc18-1+/−) and mice overexpressing Munc18-1 (Munc18-1OE) (Toonen et al., 2006b). A clear positive correlation between release parameters in the NMJ and the level of Munc18-1 was described. In the present study, we investigated the role of Munc18-1 in homeostatic upregulation of transmitter release in the NMJ. Heterozygous Munc18-1 null-mutant mice and mice overexpressing Munc18-1 were subjected to an experimental form of MG involving chronic in vivo application of the AChR blocker α-bungarotoxin and it was investigated whether compensatory increase of ACh release occurred at their NMJs. Our results indicate that Munc18-1 is indeed involved in this form of synaptic adaptation.

Materials and Methods

Mice

Munc18-1+/− mice were bred by means of standard mouse husbandry and backcrossed for at least 6 generations to a C57Bl/6 background (Verhage et al., 2000). These mice had 50% reduced Munc18-1 protein level in brain, as determined by Western blot analysis (Toonen et al., 2006b).

Munc18-1OE mice were generated as described (Toonen et al., 2006b). The linearized pNSE-Ex4 minigene containing the promoter of neuron specific enolase from rat (Forss-Petter et al., 1990) and Munc18-1 cDNA was injected into zygotes with a C57Bl/6 x CBA background. The transgenic mice were viable and fertile. Histological analysis of brain and spinal cord sections at C1-C3 level, from which the innervation of the diaphragm muscle originates, showed transgene expression. In the spinal cord, the transgene staining was co-localized with staining for the motor neuronal marker choline-acetyltransferase.

Genotypes were analyzed using Southern blot or polymerase chain reaction methods. The mice were housed under a 12 h light / 12 h dark regime with ad libitum food and water. The animals were euthanized by CO₂ inhalation. Hemi-diaphragms with their phrenic nerves were rapidly dissected and pinned out in Ringer’s solution (see below) at room temperature.

All animal experiments were carried out according to the Dutch law and local University guidelines (DEC#99029).

Toxin-induced myasthenia gravis

We used an experimental animal model for MG that was originally developed in rats and is termed toxin-induced MG (TIMG) (Molenaar et al., 1991). The model involves repeated injections with small doses of α-bungarotoxin (Biotoxins Incor-
porated, ST. Cloud, FA, USA), a highly potent and selective blocker of AChRs at NMJs. We adjusted and re-evaluated the model for use with mice (data not shown).

During a period of four weeks, mice of about 4 months of age (body weights of about 20 g) were injected intraperitoneally every 48 hour during the work week (i.e. on Mondays, Wednesdays and Fridays) with a low dose of \( \alpha \)-bungarotoxin. Control groups received physiological saline (0.9% NaCl) injections. As a first dose, 1.2 \( \mu g \) \( \alpha \)-bungarotoxin was injected. Thereafter, doses of 0.8 \( \mu g \) were given. Three hours after an injection the mice showed slightly invaginated flanks, indicating mild muscle weakness. There were no apparent breathing problems. In general, these symptoms had disappeared by the next, toxin-free, day. If the symptoms of weakness (scored in individual mice) were too severe, the dose of \( \alpha \)-bungarotoxin was lowered. The mice were well able to feed and drink. At the end of the TIMG treatment, body weights of the TIMG and saline-injected control mice were similar.

Neuromuscular synapse electrophysiology

Measurements were performed on nerve/muscle preparations from right and left hemi-diaphragms of wild type, Munc18-1\(^{+/+}\), and Munc18-1\(^{OE}\) mice. Upon stimulation of the phrenic nerve, the nerve terminal releases a number of ACh quanta. Part of the transmitter binds postsynaptic ACh receptors and causes them to open. The resulting ion current gives rise to an endplate potential (EPP). Spontaneous release of single quanta results in miniature EPPs (MEPPs). We recorded EPPs and MEPPs by impaling muscle fibers near the NMJ with a 10-20 MΩ glass capillary microelectrode filled with 3 M KCl and standard recording equipment at 26-28°C (Plomp et al., 1994) in Ringer’s medium containing (mM): NaCl, 116; KCl, 4.5; MgCl\(_2\), 1; CaCl\(_2\), 2; NaH\(_2\)PO\(_4\), 1; NaHCO\(_3\), 23; glucose 11; pH 7.4, gassed with 95% O\(_2\)/5% CO\(_2\). Hemidiaphragms were incubated with 3.1 \( \mu M \) µ-Conotoxin GIIIB (Scientific Marketing Associates, Herts, UK), a selective blocker of muscle sodium channels, to prevent action potentials. This allowed for the undisturbed recording of EPPs during electrical nerve stimulation (0.3 and 40 Hz) of the phrenic nerve with a bipolar stimulation electrode. The quantal content (i.e. the number ACh quanta released upon a single nerve impulse) at each NMJ was calculated from EPP and MEPP amplitudes, normalized to -75 mV membrane potential. The EPP amplitude was corrected for non-linear summation, as described before (Plomp et al., 1994). During high-frequent stimulation (1 s, 40 Hz), EPP amplitudes decrease to a plateau level. The rundown level of the EPP amplitude during 40 Hz stimulation for 1 s of the phrenic nerve was determined by averaging the amplitudes of the last 10 EPPs of the train and expressing it as percentage of the amplitude of the first EPP of the train.

A GeneClamp 500B amplifier from Axon Instruments (Union City, CA, USA) was used for amplifying and filtering (10 kHz low-pass) of the signals. The record-
ings were digitized and analyzed using a Digidata 1200 interface, Clampex 8.0 and Clampfit 8.0 programs (Axon Instruments) and routines programmed in Matlab (The MathWorks Inc., Natick, MA, USA).

Statistical analysis
The significance of differences in mean values between genotype groups were tested using Student’s t-test. Differences in effect of TIMG treatment between genotypes was tested with the multivariate general linear model (interaction parameter) and the post-hoc Tukey test by using SPSS 10.0 for Windows (SPSS Inc., Chicago, IL, USA).

Results
Analysis of basic neuromuscular synaptic function in Munc18+/- mice
We performed intracellular recordings of EPPs from muscle fibers of diaphragm-phrenic nerve-muscle preparations from wild type mice (N=12, 10-15 NMJs sampled per animal) and Munc18-1+/- mice (N=10, 10-15 NMJs sampled per animal). These mice had received repeated injections with saline and served as a control for the TIMG group that was injected with α-bungarotoxin (see below).

The MEPP amplitude was the same in both genotype groups (0.93 ± 0.05 and 0.93 ± 0.05 mV, Figure 1A, G). We did not observe differences in spontaneous quantal ACh release between wild type and Munc18-1+/- NMJs. The mean MEPP frequency was 1.62 ± 0.10 and 1.52 ± 0.10 s⁻¹, respectively (Figure 1B, G). At low-frequency nerve stimulation (0.3 Hz), the mean amplitude of the EPPs was 25.66 ± 1.31 mV in wild type and 25.44 ± 1.17 mV in Munc18-1+/- NMJs. The calculated quantal content was 41.54 ± 2.08 in wild type and 40.57 ± 2.31 in Munc18-1+/- NMJs (Figure 1C). Upon high-frequency (40 Hz) nerve stimulation, Munc18-1+/- NMJs displayed a more pronounced rundown of EPP amplitude, to a level of 74 ± 1.3% of the amplitude of the first EPP of the stimulation train, compared to 83 ± 0.9% at wild type NMJs (Figure 2, p<0.001).

Thus, although low-frequency evoked ACh release as well as spontaneous release did not differ between Munc18-1+/- and wild type NMJs, a more pronounced rundown of EPP amplitude was observed at Munc18-1+/- NMJs at high-frequency nerve stimulation. This most likely reflects a decrease in quantal content during the stimulus train.

Myasthenic Munc18-1+/- neuromuscular synapses show impaired compensatory increase of ACh release
Subjection of mice to the TIMG protocol (four weeks α-bungarotoxin treatment) induced a reduction of the mean MEPP amplitude of about 65% at NMJs of both
Figure 1. Low-rate evoked and spontaneous acetylcholine release at neuromuscular synapses of wild type, Munc18-1+/− and Munc18-1OE mice with toxin-induced myasthenia gravis. Effect of TIMG-treatment (shaded bars) on MEPP amplitude, quantal content at 0.3 Hz nerve stimulation and MEPP frequency at NMJs of diaphragms from Munc18-1+/− (A–C), Munc18-1OE (D–F) mice, compared with wild type littermates. N=5–12 mice, 10–15 NMJs sampled per muscle, data represent grand mean group values + S.E.M. TIMG-treatment induced equal reductions of MEPP amplitudes of ~70% in either genotype. The extent of the resulting increase in quantal content in the wild type group was larger than that in the Munc18-1+/− group (p<0.05). MEPP frequency at saline-injected control Munc18-1OE NMJs was higher than at wild type NMJs (p<0.05). TIMG treatment reduced the MEPP frequency at Munc18-1OE NMJs (p<0.05), to a larger extent than at wild type NMJs (P<0.01). (G) Typical examples of MEPP recordings at NMJs of the different genotype mice under TIMG- and saline-injected control condition (10s traces, left and right columns). In the middle column, individual MEPPs recorded from control and TIMG-treated NMJs have been superimposed at enlarged time-base. *p<0.05, **p<0.001.
Munc18-1 TIMG

Figure 2. High-rate evoked acetylcholine release at neuromuscular synapses of wild type, Munc18-1\(^{+/−}\) and Munc18-1\(^{OE}\) mice with toxin-induced myasthenia gravis.

Effect of TIMG-treatment (shaded bars) on EPP amplitude rundown during high-rate (40 Hz) stimulation for 1 s of the phrenic nerve of diaphragms from wild-type, Munc18-1\(^{+/−}\) and Munc18-1\(^{OE}\) mice (N=5-12 mice, 10-15 NMJs sampled per muscle). (A) Mean rundown level of EPP amplitude. At each NMJ the EPP amplitude rundown level was taken as the calculated mean amplitude of the last 10 EPPs in the train, expressed as a percentage of the first EPP of the train. Data represent grand mean group values ± S.E.M. Average rundown profiles from (B) wild-type, (C) Munc18-1\(^{+/−}\) and (D) Munc18-1\(^{OE}\) NMJs under saline-injected control condition (filled circles) and TIMG condition (open circles). Insets are typical examples of 40 Hz EPP train recordings. ***p<0.001.

Munc18-1\(^{+/−}\) and wild type mice, to 0.34 ± 0.01 mV (n=11) and 0.31 ± 0.02 mV (n=10), respectively (Figure 1A, G). This indicates that the reduction of postsynaptic sensitivity to ACh in the TIMG condition was established at a similar degree in both genotypes. The amplitude of EPPs evoked by low-frequency stimulation (0.3 Hz) after TIMG treatment was 19.5 ± 1.43 mV in wild type and 17.8 ± 0.64 mV in Munc18-1\(^{+/−}\) NMJs. At wild type TIMG NMJs the quantal content was increased by 126% (p<0.001) compared with the saline-treated controls (Figure 1C). However, the increase of quantal content at Munc18-1\(^{+/−}\) TIMG NMJs was only 79% (p<0.001), statistically significantly less than the increase at wild type NMJs (p<0.01). Thus, although basic neuromuscular synaptic transmission seems largely unaltered at Munc18-1\(^{+/−}\) NMJs, presynaptic adaptation in response to reduced postsynaptic transmitter sensitivity is impaired.

In both wild type and Munc18-1\(^{+/−}\) groups, rundown of the EPP amplitude at 40 Hz stimulation was more pronounced in TIMG than saline-injected control mice (Figure 2). In wild type TIMG-NMJs the rundown reached a level of 62 ± 2.4% of
the first EPP in the train, which differed from the 83 ± 0.9% observed in controls (p<0.001). In Munc18-1+/− TIMG NMJs the rundown of EPPs amplitude increased to 59 ± 1.8%, which was significantly different from control values (74 ± 1.3%, p<0.001).

At TIMG-NMJs the mean MEPP frequency tended to be reduced in both wild type (9%) and Munc18-1+/− mice (18%), compared with their saline-injected controls, but this difference was not statistically significant. At NMJs with very small MEPPs, as often encountered under TIMG conditions, the mean frequency may have been underestimated due to loss of MEPPs that were too small to detect, i.e. with amplitudes smaller than about 0.1 mV. In order to substantiate this hypothesis we classified the NMJs with respect to their MEPP amplitude into classes of 0.1 mV bin-width and calculated for each class the mean MEPP frequency. In all overlapping MEPP amplitude classes there was no statistically significant difference between MEPP frequencies measured in TIMG or control NMJs, in either wild type or Munc18-1+/− NMJs (Figure 3). The wild type and Munc18-1+/− TIMG-NMJs in the 0.1–0.2 mV MEPP amplitude class (which were not encountered in saline-treated controls) had clearly lower MEPP frequencies than all the classes of NMJs, with larger MEPP amplitude. This indicates that some loss of MEPPs in the baseline noise had occurred, which may explain the small reduction of overall mean MEPP frequency in the TIMG groups of either genotype compared to their control value obtained in saline-injected mice. As a result the mean MEPP amplitude may have been overestimated at these NMJs and, consequently, the TIMG quantal contents may have been underestimated.

Figure 3. Relation between MEPP amplitude and MEPP frequency at neuromuscular synapses of Munc18-1+/− and Munc18-1OE mice with toxin-induced myasthenia gravis.
NMJs of (A) wild-type, (B) Munc18-1+/− and (C) Munc18-1OE mice were classified with respect to their mean MEPP amplitudes (normalized to -75 mV resting membrane potential) in bins of 0.1 mV width. Data points represent the mean MEPP frequency ± S.E.M. within a bin (open symbols, TIMG; filled symbols, saline-injected controls). In the lower panels of each figure, the numbers of NMJs in each bin are given in a histogram (filled bars, TIMG; open bars, saline-injected control). No statistically significant differences were found between TIMG and controls NMJs, when both present in a bin.
Thus, NMJs from Munc18-1\(^{+/−}\) mice showed impaired synaptic plasticity in that they were not able to upregulate their ACh release as much as wild type control NMJs upon reduction of postsynaptic transmitter sensitivity (Figure 4).

**Overexpression of Munc18-1 does not further increase the level of homeostatic upregulation of transmitter release in TIMG**

The data obtained from Munc18-1\(^{+/−}\) mice indicated that a reduced level of Munc18-1 limits the ability of motor nerve terminals to compensate for the reduced postsynaptic ACh sensitivity under myasthenic conditions by an increase of ACh release. Consequently, we hypothesized that increased Munc18-1 protein levels might increase the level of compensatory upregulation of ACh release. To test this hypothesis we subjected Munc18-1\(^{OE}\) mice, which overexpress Munc18-1 in motor neurons, to our TIMG protocol and analyzed synaptic transmission at their NMJs.

Under control (i.e. saline-injected) conditions (N=7 wild type mice and N=5 Munc18-1\(^{OE}\) mice, 10-15 endplates per muscle), MEPP amplitude was similar at wild-type and Munc18-1\(^{OE}\) NMJs (0.99 ± 0.09 mV and 0.90 ± 0.04, respectively, Figure 1E, G). Munc18-1\(^{OE}\) NMJs had a 36% increased MEPP frequency compared with wild type (2.21 ± 0.17 s\(^{−1}\) and 1.62 ± 0.14 s\(^{−1}\), respectively, p<0.05, Figure 1E, G). The mean quantal content of Munc18-1\(^{OE}\) NMJs was 46.6 ± 1.05, which is 18% more than the 39.4 ± 2.68 observed at wild type NMJs, although this difference

![Figure 4. Normalized effects of toxin-induced myasthenia gravis on neuromuscular synapse parameters of wild type, Munc18-1\(^{+/−}\) and Munc18-1\(^{OE}\) mice.](image)

Overview of the effects of TIMG-treatment on MEPP amplitude, MEPP frequency, quantal content and rundown of 40 Hz EPP amplitude in wild-type, Munc18-1\(^{+/−}\) and Munc18-1\(^{OE}\) NMJs. TIMG values of the parameters of each genotype group were expressed as a percentage of the mean values obtained in saline-injected control groups. *p<0.05, different from wild type group.
did just not reach statistical significance \( (p=0.05, \text{ Figure 1F}) \). Also, there was no significant change in EPP amplitude rundown upon 40 Hz stimulation at Munc18\(^{\text{OE}}\) NMJs compared to wild type (rundown to 80 ± 0.7% and 82 ± 0.7% of the first EPP, respectively, Figure 2).

At Munc18-1\(^{\text{OE}}\) NMJs under TIMG conditions there was a tendency to display less compensatory increase of ACh release than at TIMG/wild type NMJs (78% vs. 121% increase, respectively, \(N=5\) TIMG/Munc18-1\(^{\text{OE}}\) and 5 TIMG/wild type mice, Figure 1F). However, this difference was not statistically significant. Under TIMG conditions, the EPP amplitude rundown became more pronounced, compared to saline-injected controls, to a similar degree in both genotypes (rundown level of 61 ± 3.6% of first EPP at TIMG/wild type and 65 ± 2.4% at TIMG/Munc18-1\(^{\text{OE}}\) NMJs, Figure 2). We observed a TIMG-induced decrease of the overall mean MEPP-frequency of 44% at Munc18-1\(^{\text{OE}}\) NMJs, which was larger \((p<0.05)\) than the small TIMG-induced decrease of ~10% at wild type NMJs (Figure 1E, G). When we classified the NMJs in MEPP amplitude bins of 0.1 mV it appeared that there was a clear tendency of MEPP frequencies at TIMG/Munc18-1\(^{\text{OE}}\) NMJs to be about 50% lower than that of NMJs in the similar amplitude class of saline-injected controls. However, these reductions were not statistically significant (Figure 3 and 4).

Thus, overexpression of Munc18-1 did not further augment the level of compensatory increase of ACh release upon a reduction in postsynaptic ACh sensitivity.

**Discussion**

At myasthenic NMJs, homeostatic upregulation of ACh release partially compensates for the loss of postsynaptic transmitter sensitivity after reduction of AChRs. Most likely, this involves retrograde signaling from muscle fibers to motor nerve terminals. We hypothesized that neuro-exocytotic proteins are the final targets of such a signaling pathway. The data presented here indicate that the key neuro-exocytotic protein Munc18-1 indeed plays a role in the mechanism underlying this form of functional synaptic plasticity.

**Role of Munc18-1 in neurotransmitter release at the neuromuscular synapse**

Munc18-1\(^{+/−}\) saline control group NMJs showed increased rundown of EPP amplitude during high-frequent stimulation, compared to wild-type, and Munc18-1\(^{\text{OE}}\) NMJs displayed an increased MEPP frequency. This is in agreement with data we reported previously on NMJs and hippocampal autaptic synapses of Munc18-1\(^{+/−}\) and Munc18-1\(^{\text{OE}}\) mice (Toonen et al., 2006b), concluding a role for Munc18-1 in regulation of the vesicle pool size. A smaller vesicle pool size in Munc18-1\(^{+/−}\) NMJs explains the reduced MEPP frequency, since vesicle pool size is a factor that de-
termines the level of spontaneous transmitter release (Prange and Murphy, 1999; Stevens and Sullivan, 1998). Rundown of high-rate evoked transmitter release is believed to represent depletion of the readily releasable pool of synaptic vesicles (Kuromi and Kidokoro, 2000), and Munc18-1 also controls the number of docked, release-ready vesicles in chromaffin cells (Toonen et al., 2006a; Voets et al., 2001) and C. elegans (Weimer and Jorgensen, 2003). The increased rundown of high-rate evoked release as observed in Munc18-1+/− is consistent with these reports.

Our earlier studies on Munc18-1+/− NMJs showed a decrease in quantal content and MEPP frequency, which we did not find in the present study. In addition we observed an increase in quantal content in Munc18-1OE NMJs and in MEPP frequency in in Munc18-1OE NMJs (Toonen et al., 2006b). Although we did find the increase in MEPP frequency, the increase in quantal content in this study was not statistically significant. The reason for this discrepancy is not clear but might stem from the difference in age of the mice. The mice used in the present study were older due to the TIMG/saline treatment of six weeks. Possibly, adaptation occurs to the changed Munc18-1 levels during aging.

Role of Munc18-1 in homeostatic transsynaptic upregulation of transmitter release at myasthenic neuromuscular synapses

Subjection of Munc18-1+/− mice to an experimental model for MG showed that these NMJs are less capable of compensatory increase of transmitter release compared to wild-type myasthenic NMJs. This indicates that Munc18-1 is involved in the homeostatic upregulation of ACh release at myasthenic NMJs.

It has been shown that Munc18-1 is involved in all steps leading to neurotransmitter release, from tethering, docking, priming to fusion (Rizo and Rosenmund, 2008; reviewed in: Toonen and Verhage, 2007). Munc18-1 may act as crystallization points for SNARE formation, so recruitment or activation of Munc18-1 may lead to an increased number of ACh quanta released following a presynaptic impulse. This may be the result of an increase of the readily releasable pool (RRP) of vesicles. This is in accordance with the observed more pronounced rundown during high-frequency stimulation in TIMG and MG, since the maintenance of a high vesicle turnover in such a large RRP during high-frequency stimulation will put more strain to the exocytotic machinery. However, one could expect a higher basal MEPP frequency in TIMG NMJs when the RRP is increased, which was not observed.

Munc18-1 is a substrate for cyclin-dependent kinase 5 (Fletcher et al., 1999; Shuang et al., 1998) and protein kinase C (PKC) (Barclay et al., 2003; de Vries et al., 2000; Fujita et al., 1996). Activation of PKC has been shown to underlie enhancement of neurotransmitter secretion in different forms of synaptic plasticity (Brager et al., 2003). Phosphorylation of Munc18-1 might be involved in the upregulation of ACh release at myasthenic NMJs. This would require stimulation of kinases at
motor nerve terminal by the putative retrograde messenger that is released from the myasthenic muscle fiber. Interestingly, stimulation of TrkB membrane receptors by neurotrophins, which are candidate retrograde factors, has been shown to activate PKC (Zirrgiebel et al., 1995). However, other studies did not find an effect of PKC inhibition on upregulated ACh release in rat TIMG NMJs in vitro (Plomp and Moelenaar, 1996). In addition, PKC phosphorylation of Munc18-1 is known to stimulate release in chromaffin cells (Barclay et al., 2003), without increasing the number of docked vesicles (Wierda et al., 2007).

Another mechanism by which Munc18-1 might be targeted in the signaling cascade is via a transsynaptic protein complex formed by the postsynaptic scaffolding protein PSD-95, and neuroligin and presynaptic β-neurexin (Futai et al., 2007). This signaling route was described for rat hippocampal CA1 pyramidal neurons, and positively influences presynaptic release. Since β-neurexin binds to the scaffolding protein CASK, which in turn is known to bind Mint1, a binding partner of Munc18-1, a role of Munc18-1 could be envisioned here (Futai et al., 2007, and references herein). However, more research is necessary to elucidate the exact signaling pathway that is employed in MG synapses.

Surprisingly, Munc18-1 OE NMJs did not show further enhanced levels of upregulation of ACh release upon the loss of postsynaptic ACh sensitivity induced by the TIMG treatment, when compared to the augmented level at wild type TIMG-treated NMJs. Upregulation of ACh release rather tended to be lower in Munc18-1 OE TIMG-NMJs, but the difference was not significant. Apparently, Munc18-1 protein at Munc18-1 OE NMJs is present at a supramaximal level and under this condition other factors that participate in the mechanism of upregulation of ACh release will become limiting. Alternatively, Munc18-1 at this increased level may exert unknown inhibitory actions on such factors.

TIMG treatment reduced MEPP frequency at Munc18-1 OE NMJs but not at wild type controls. The reasons for this difference are not clear. One possible explanation is that a larger proportion of MEPPs remained undetected due to a larger reduction of MEPP amplitude at Munc18-1 OE NMJs by the TIMG treatment. However, this was not observed. It is possible that due to the already elevated ACh release level at Munc18-1 OE NMJs, compared to wild type controls, a further increase of evoked release induced by the TIMG treatment prevents full replenishment of the transmitter vesicle pool so that MEPP frequency becomes reduced.

TIMG induced a more pronounced rundown of ACh release at high-rate nerve stimulation at NMJs of all three studied Munc18-1 genotypes, compared to that at NMJs of saline-injected control mice. As the quantal content is increased under TIMG conditions, the most likely explanation is that the readily releasable vesicle pool becomes exhausted at a faster rate. A similar association between increased quantal content and a more pronounced rundown at high-rate nerve stimulation
was shown at NMJs of mice overexpressing acetylcholinesterase (Farchi et al., 2003). However, increased exhaustion of the vesicle pool due to the high level of ACh release may not be the only explanation because an increased rundown of release has been observed as soon as 3 hours after the first α-bungarotoxin injection in TIMG rats, when MEPP amplitude is decreased but quantal content not yet increased (J.J. Plomp, unpublished results). Furthermore, it was expected that increased levels of Munc18-1 at Munc18-1\textsuperscript{OE} NMJs would have counteracted more pronounced rundown of ACh release during high-rate nerve stimulation through facilitation of the replenishment rate of the vesicle pool. However, this was not observed; apparently Munc18-1 is not the rate-limiting factor in this process.

In conclusion, we have shown that Munc18-1 is involved in homeostatic upregulation of ACh release at mouse NMJs in response to a lowered postsynaptic sensitivity for ACh. From a clinical point of view, elucidation of the molecular mechanism of upregulation of ACh release at myasthenic NMJs is important. New drug targets can be identified and their activation should boost the process of synaptic adaptation in order to counteract or prevent muscle weakness. Unfortunately, Munc18-1 appears to be a poor candidate in view of the observed lack of enhanced compensatory upregulation of ACh release at myasthenic Munc18-1\textsuperscript{OE} NMJs.

Reference List


