Summary and General Discussion
Aim

The aim of the studies described in this thesis was to elucidate the roles of several neuro-exocytotic proteins at the motor nerve terminal in neuromuscular synaptic transmission, making use of genetic knockout (KO) mice, each missing one (or more) neuro-exocytotic proteins. In addition, it was investigated in a pharmacological mouse model for myasthenia gravis (MG) whether some of these proteins play a role in the phenomenon of compensatory upregulation of acetylcholine (ACh) release at the neuromuscular junction (NMJ) in this disease. This chapter briefly summarizes the major findings of this thesis and addresses some of the remaining questions. In addition, suggestions for future research are made.
Summary of experimental chapters

Chapter 2 describes the electrophysiological and morphological analysis of NMJs from mice lacking Munc13-1 and Munc13-2. Munc13 has been implicated in the priming of synaptic vesicles. In contrast to Munc13-1 and Munc13-2 single KOs, the double KO mice are not viable and die upon birth. Therefore, NMJs of these mice were investigated at embryonic day 18.5. Although evoked release was strongly reduced in the NMJ of Munc13-1/2 deficient mice, miniature endplate potentials (MEPPs, i.e. spontaneous ACh release) were observed at more than 2-fold higher frequency, compared to wild-type. This was surprising, because hippocampal synapses in these mice have been reported to be completely silenced. The current finding thus suggests that vesicle priming at the NMJ is partly Munc13 independent. Morphological analysis of the diaphragm showed a highly disorganized muscle fibre orientation and endplates that were no longer organized in the typical mid-line endplate-band as observed in wild-type diaphragms. This phenotype closely resembled that of the choline acetyltransferase KO mice (Brandon et al., 2003), at which NMJs are completely silenced, indicating that action potential evoked ACh release is necessary for normal muscle development. The current findings at Munc13-1/2 double KO mice indicate that the presence of spontaneous ACh release (MEPPs) alone is not sufficient for normal muscle development.

In chapter 3, the role of α-neurexins in the NMJ was investigated. There are three isoforms, α-neurexin 1, -2, and -3 (for review, see Missler and Sudhof, 1998). α-Neurexins are transmembrane proteins with an intracellular domain enabling interaction with components of the release machinery, and an extracellular domain that can participate in a transsynaptic protein complex. Deleting α-neurexins in mice results in strongly reduced neurotransmitter release in CNS (Missler et al., 2003). α-Neurexins presumably regulate release through interaction with the Ca_{v2.2} and Ca_{v2.1} voltage gated Ca^{2+} channels (Zhang et al., 2005). Mice lacking all three α-neurexin isoforms die at birth, but a subset of mice lacking two isoforms survives to adulthood. We used these mice to analyze NMJ function in two different muscle types (the slow-twitch fiber soleus muscle, and the diaphragm with a mixture of slow and fast twitch fibers). Neuromuscular synapse function in the soleus muscle was slightly impaired, showing a decrease in both MEPP frequency and quantal content of about 30%. In diaphragm NMJs, however, no effect was observed. To investigate the release characteristics of a NMJ without any α-neurexin present, we examined the embryonic diaphragm NMJ of triple KO mice and found undisturbed transmitter release, too. To determine a possible role of α-neurexins in synapse homeostasis, we investigated α-neurexin double KO mice under the condition of toxin-induced MG (TIMG), a model for MG using α-bungarotoxin. This condition normally results in ~2-fold upregulation of evoked ACh release at the NMJ, partially com-
pensating the reduced density of functional ACh receptors. Indeed, compensatory upregulation of quantal content at the α-neurexin double KO NMJ was much less pronounced than in the wild-type controls. These findings show that α-neurexins are important for efficient neurotransmitter release at some types of NMJs and that they play a role in the mechanism of synaptic homeostasis at NMJ under myasthenic condition.

Rab3A is one of a family of small GTP-binding proteins associated with synaptic vesicles, which have been implicated in modulating evoked neurotransmitter release in brain synapses (Fischer von Mollard et al., 1991; Geppert et al., 1994; Fischer et al., 1994; Star et al., 2005). Compound KO mice for Rab3A, Rab3B, Rab3C, and Rab3D are not viable (Schluter et al., 2004). Chapter 4 describes the NMJ function in soleus and diaphragm of Rab3A deficient mice. We found 20-40% reduction in spontaneous ACh release (MEPP frequency) but unaltered nerve action potential-evoked release and recovery from release induced by high-frequency stimulation. This indicates a selective role of Rab3A in spontaneous transmitter release at the NMJ that cannot or only partially be taken over by the closely related Rab3B, -C, or -D isoforms. It has been hypothesized that Rab3A mutation underlies ACh release deficits in human presynaptic myasthenic syndromes. Our observation of a normal level of evoked ACh release at the Rab3A KO NMJ, however, argues against this hypothesis.

Chapter 5 describes the effect of varying Munc18-1 protein levels in cultures of hippocampal neurons and the NMJ. Munc18-1 is the only protein described in this thesis whose deletion is lethal and causes a total arrest of neurotransmitter release in CNS and PNS (Verhage et al., 2000). Heterozygous Munc18-1 KO (Munc18+/-) mice are viable and have a 50% reduction of Munc18-1 protein level in the brain, making them a suitable model to study the effect of decreased Munc18-1 level. In addition, the effect of increased level of the protein on exocytosis was studied in Munc18-1 overexpressing mouse (Munc18OE) and in hippocampal cell cultures overexpressing the protein after viral transfection. NMJ function was analyzed in wild-type mice, Munc18+/-, and Munc18OE mice. A clear 'gene-dosage' effect of Munc18-1 was observed on MEPP frequency and quantal content. In Munc18+/- NMJs, increased run-down of ACh release upon high-frequency stimulation was observed as well, and a decreased response to a hypertonic shock, used to probe for the size of the readily releasable pool of synaptic vesicles. Electrophysiological synaptic analysis of hippocampal neuron cultures from Munc18-1+/- mice showed a similar increased run-down of transmitter release during high-frequency stimulation and decreased response to hypertonic stimulation. Viral overexpression of Munc18-1 in these cultured hippocampal synapses, on the other hand, increased the response to hypertonic shock and resulted in less pronounced run-down during high-frequency stimulation. Electron microscopic analy-
sis showed a clear difference in the number of morphologically docked vesicles. These data led to the conclusion that Munc18-1 is involved in modulating the number of docked, release-ready synaptic vesicles at nerve terminals.

**Chapter 6** describes a study where Munc18-1\(^{\text{OE}}\), Munc18-1\(^{+/−}\), and wild-type mice were subjected to the TIMG model. It appeared that Munc18-1\(^{+/−}\) NMJs were less able to increase the quantal content than wild-type NMJs. Overexpression of Munc18-1 on the other hand failed to increase the compensatory response. These results indicate that Munc18-1 is involved in the signaling cascade leading to increased quantal content in TIMG but that other factors may become rate-limiting in the Munc18-1\(^{\text{OE}}\) NMJ.

RIM1α is a known binding partner of Rab3A (Wang et al., 2000) and has been suggested to play a role in synaptic vesicle priming. Mice in which RIM1α is deleted are viable and show minor behavioral defects (Castillo et al., 2002; Schoch et al., 2002; Powell et al., 2004; Calakos et al., 2004). **Chapter 7** describes that deleting RIM1α in mice does not affect NMJ function, neither under basal conditions, nor in the condition of TIMG. Most likely, RIM1α is redundant and RIM2α assumes its function. Indeed, recent data show that RIM1α/2α double KO mice (Schoch et al., 2006) die upon birth and their embryonic NMJs show strongly impaired evoked ACh release.

### Phenotypic differences between central and neuromuscular synapses of neuro-exocytotic protein knockout mice

This thesis has contributed to the knowledge on the role of Munc18-1, Munc13s, Rab3A, Neurexins and RIM1α in neuromuscular synaptic transmission. When comparing the function of these proteins in NMJ and CNS, it is interesting that usually the synaptic phenotype in the NMJ is much milder compared to that in synapses of the CNS. The strongest example is Munc13, deletion of which results in complete ablation of neurotransmitter release in CNS (Varoqueaux et al., 2002) while the NMJ still exhibits MEPPs and even, to some extent, retains nerve-stimulated release. Deleting Rab3A and α-neurexin also has a much milder effect on release properties of the NMJ compared to CNS, while deleting RIM1α failed to elicit any effect. For Munc18-1, the overall similarity between CNS and NMJ phenotype was the strongest. Munc18-1 is the only protein among the ones investigated here whose deletion in mice results in lethality; the two remaining isoforms are apparently not able to take over its functions. Thus, Munc18-1 is essential for neurotransmission at the NMJ, while the other proteins fulfill a more regulatory function, or can be compensated for by isoforms or other proteins.

The NMJ is different from synapses in the CNS both in function and morphology, and therefore it may not be surprising that this is reflected in the molecular organization of the secretion machinery. In contrast to CNS synapses, the post-
synaptic cell is not another neuron, the number of vesicles that is released in an action potential dependent manner is much higher (diaphragm NMJ: around 30; CNS synapses: around 3), the probability of release in the NMJ is approximately 100% and there is only one type of neurotransmitter. In addition, although plasticity is employed to ensure successful muscle contraction, plasticity in the CNS plays a much more crucial role and forms the basis of many of the brain’s functions like learning and memory. Concluding, the role of a certain protein in the process of secretion is depending on the cellular context in which it is exerting its role.

**Role of the studied neuro-exocytotic proteins in synaptic homeostasis and relevance to myasthenia gravis**

One of the goals of this thesis was to investigate the role of specific presynaptic proteins in the homeostatic upregulation of ACh release as observed in NMJs of muscle biopsies from MG patients, by applying the TIMG model in mice genetically lacking these proteins. The phenomenon of compensatory increase of transmitter release has been described in several other experimental systems (see Introduction). The decreased sensitivity to ACh due to a lowered density of postsynaptic ACh receptors leads via a largely unknown mechanism to increased ACh release from the presynaptic nerve terminal. Proteins that are playing a role in transmitter release are likely targets of this signaling mechanism. We investigated the effect of deleting Munc18-1, Neurexins and RIM1α on the homeostatic response at the myasthenic NMJ.

**Munc18-1**

In NMJs with only 50% Munc18-1 expression level, the compensatory response in TIMG was less pronounced arguing for the implication of this protein somewhere in the signaling pathway. Munc18-1 has been implied in regulating the readily releasable pool of vesicles, which may be the mechanism of increase of ACh release in TIMG. However, when we exposed NMJs from TIMG muscles to high-sucrose medium to probe for the size of the pool we failed to see an increase, rendering it unlikely that it is this specific function of Munc18-1 that is modulated for increase in ACh release. This raises the possibility that instead of being an active player in the signaling cascade, Munc18-1 is present in rate-limiting amounts in the strained exocytotic machinery in NMJs of Munc18-1 heterozygous mice. In line with this, increasing Munc18-1 levels in NMJs had no effect on the homeostatic performance of the synapse under TIMG conditions.

**α-Neurexins**

Mice lacking two of the three isoforms of α-neurexin showed impaired upregulation of transmitter release at the TIMG NMJ. Interestingly, diaphragm NMJs from
neurexin double KO mice did not differ in ACh release characteristics from wild-type under basal conditions. Even the release properties of NMJs from (embryonic) mice lacking all three isoforms of neurexin were unchanged, indicating that this protein is not involved in basic neurotransmitter exocytosis at the NMJ. The decreased upregulation observed in TIMG neurexin double KO NMJs thus indicates that neurexin is specifically involved in plasticity of the diaphragm NMJ, possibly quite far upstream the exocytotic processes, because neurexin has been suggested to participate in trans-cellular signaling processes. Alternatively, neurexin’s role in the homeostatic response may lay in modulation of the coupling of calcium channels with the exocytotic machinery, as observed in central synapses.

**RIM1α**

Although we did not observe functional changes in NMJs lacking RIM1α (neither under basal nor TIMG conditions) we cannot exclude a role of RIM proteins in homeostasis at the NMJ, because RIM1/2α double KO mice have impaired ACh release at the NMJ, and die perinatally (Schoch et al., 2006). This indicates that Rim2α takes on the role of RIM1α in the RIM1α KO mouse. To further investigate RIM protein involved in synaptic homeostasis at the NMJ, it would be interesting to apply the TIMG model on mice homozygous for RIM1α and heterozygous for RIM2α.

One can wonder whether it is possible to really pinpoint essential factors in the signaling cascade that leads to increased neurotransmitter release in TIMG NMJs. Homeostatic processes are crucial to the survival of the organism, and it is not unthinkable that evolution has produced many parallel pathways and players to ensure NMJ transmission.

The clinical relevance for MG of this rather fundamental neurobiological study is yet unclear. It may be that myasthenic patients exist with a certain polymorphism/splice variant for munc18-1 or neurexin, causing them to have less compensatory upregulation of transmitter release. These patients may have more severe paralytic symptoms. More research into the details of pathways leading to the NMJ’s ability to counteract threats to signal transmission may be beneficial for many more diseases in which successful neurotransmission is compromised, like Lambert Eaton myasthenic syndrome and congenital myasthenic syndromes (see for review Boonyapisit et al., 1999). Furthermore, such studies may reveal new pharmacological targets that may be exploited to increase ACh release to overcome or prevent block of neuromuscular transmission.

**Considerations on the use of knockout mice**

Experiments described in this thesis have been carried out on genetically modified mice (mostly KO mice, and one mouse overexpressing the Munc18-1 gene). Careful
consideration of genetic background, compensatory mechanisms, homeostasis, and developmental differences is necessary when characterizing synaptic transmission in genetically engineered animals.

It is known that genes fulfill different roles during development (in utero and postnatally) and in adulthood (e.g., the dependence of NMJ synaptic transmission on different types of Ca$^{2+}$ channels (Urbano et al., 2003)).

Developmental changes due to the absence of the gene of interest may in itself cause (subtle) changes in the mechanism of synaptic homeostasis. Further activation of synaptic homeostatic mechanisms by the TIMG treatment might show an abnormal result, what could be attributed to a direct functional consequence of the absence of the KO-gene product. However, the mechanism of synaptic homeostasis might already have been altered in itself.

Furthermore, the genetic background of the mouse strain can significantly alter a neurological or musculoskeletal phenotype of transgenic animals (Yoshiki and Moriwaki, 2006). For instance, the genetic background alters the outcome of anxiety-related tasks, likely due to changes in GABAergic transmission (Robertson, 1979; Chapouthier et al., 1991; Griebel et al., 2000; Rodgers et al., 2002). Genetically deleting proteins participating in synaptic transmission may also have different effects depending on the genetic background of the mouse, as demonstrated with the serotonin-transporter (Holmes et al., 2002; Holmes et al., 2003) and serotonin receptor 5-HT1A deletions (Lesch et al., 2003), when expressed in different inbred mouse strains.

Alternatives for conventional knockout mice

In the studies of this thesis, ‘first generation’ knockout mice were used, generated by gene targeting through homologous recombination (Capecchi, 1989). One of the disadvantages of this technique is that the gene is deleted in every cell and throughout the development and lifetime of the mouse, as described above. New techniques have become available that enable temporal and spatial control of genetic manipulations: the Cre/LoxP recombinase system (Tsien et al., 1996a; Tsien et al., 1996b), and the tetracycline-inducible system (Gossen and Bujard, 1992; Furth et al., 1994). The Cre-LoxP system uses mice with LoxP sites flanking the gene of interest (floxed mice), which are bred with transgenic mice expressing Cre recombinase under the control of a selected promoter (Cre mice). Once expressed, Cre recombines the floxed gene and produces gene KO (reviewed in Gaveriaux-Ruff and Kieffer, 2007). In this system, the choice of the promoter determines the time and location of Cre expression. One possibly underestimated worry is the potential cellular toxicity of Cre recombinase (Schmidt-Supprian and Rajewsky, 2007). The tetracycline-inducible system is similar to the Cre-LoxP in that both systems make
use of homologous recombination and embryonic stem cells, and both require the
generation of two sets of transgenic mice.

Another, fundamentally different approach is RNA interference (RNAi), which
can selectively down-regulate gene expression (reviewed in Gao and Zhang, 2007).
The silencing of a gene is accomplished posttranscriptionally. Due to variation in
the degree of silencing, the term ‘knock-down’ has been introduced. The method
has been used in cell cultures and whole organisms, and the methods of delivery
can be as simple as feeding double stranded RNA to worms (Kamath and Ahringer,
2003), and by injecting RNA constructs into pronuclei. Moreover, stable RNAi
knock-down mouse have been engineered (Peng et al., 2006) although it is yet to be
confirmed that the transgenic RNAi effect is permanent (Gao and Zhang, 2007).
One drawback of the RNAi method is that there may be residual expression of
RNAi-targeted genes. In addition, there is a difference in the competency of cells to
carry out RNAi (Gao and Zhang, 2007).

RNAi has been successfully used in mammalian muscle tissue by Kong and co-
workers (Kong et al., 2004), decreasing the levels of MuSK and rapsyn. However, to
my knowledge, the method has not been used to target presynaptic proteins in the
NMJ as the the small size of the nerve terminal precludes RNAi injection.

**Outlook**

Future research on elucidating the roles of synaptic proteins in the mammalian NMJ
certainly will most likely involve the further analysis of the still growing number of
KO mice, both conventional and inducible, like described above. Subjecting these
animals to TIMG will help to gain insight into the mechanism of homeostatic in-
crease of transmitter release. It would be very helpful to induce gene KO after es-
establishment of the TIMG situation, to avoid pre-existing homeostatic mechanisms
counteracting the effects of the protein’s absence.

A publication by Das and coworkers described a co-culture of vertebrate embry-
onic motoneuron and skeletal muscle cells, which formed neuromuscular synapses
(Das et al., 2007). Although the functional aspects of these synapses were not de-
scribed in much detail in this paper, it may be interesting to use such a system to
induce TIMG. This may allow for a controlled, ex vivo experimental set-up in which
blockers can be used to investigate the homeostatic signaling cascade in detail.

RNAi sounds like a promising method as soon as it possible to knock down
genes in the nerve ending, although this method is only useful for genes that are
expressed locally.

**References**


Robertson HA (1979) Benzodiazepine receptors in “emotional” and “non-emotional” mice; comparison of four strains. Eur J Pharmacol 56: 163-166.


