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Roles of neuro-exocytotic proteins at the neuromuscular junction

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1

General Introduction

Preface

The neuromuscular junction (NMJ) connects the motor neuron and the skeletal muscle cell. In mammals, the neurotransmitter acetylcholine (ACh) is released from presynaptic terminals upon activation of the motor neuron. Subsequently, ACh will bind and activate postsynaptic ACh receptors (AChRs) leading to membrane depolarization and contraction of the muscle fiber. Proper function of the NMJ is of crucial importance for the survival of the organism. Several paralytic neuromuscular diseases are known to be caused by either post- or presynaptic NMJ dysfunction.

The NMJ has been of key importance in our understanding of chemical synaptic transmission. Since Katz and colleagues first measured synaptic events at the NMJ of frog some 60 years ago (Fatt and Katz, 1950) the NMJ has remained an important model system. It is technically easier to measure the synaptic signals in vitro at this peripheral synapse compared to synapses in the brain. Another advantage over central synapses is that it is a 'one-to-one synapse', in contrast to the central nervous system (CNS) where many presynaptic nerve terminals are present at one postsynaptic neuron. This allows for direct assessment of the physiological parameters of a single synapse.

Chemical transmission involves the highly controlled fusion of transmitter-filled vesicles with the presynaptic plasma membrane, leading to exocytosis of the transmitter into the synaptic cleft. Many presynaptic proteins important for the tight regulation of this process have been identified in the last two decades. This research has been greatly facilitated by the generation of transgenic mice lacking or over expressing specific proteins. The roles of neuro-exocytotic proteins hitherto have been experimentally characterized mainly in mouse brain slice preparations and cultured brain synapses.

This thesis investigates whether the neuro-exocytotic proteins identified in the CNS also serve this role at the presynaptic NMJ. Besides being of neurobiological importance, characterization of such roles might be relevant to primary and secondary presynaptic phenomena in diseases with NMJ dysfunction, such as (congenital forms) of presynaptic myasthenic syndromes and myasthenia gravis (MG)

The neuromuscular junction: a model-synapse

The NMJ is one of the most thoroughly studied synapses. Using the NMJ, Sir Henry Dale demonstrated the principle of chemical transmission of an electrical signal from one cell to another (Dale et al., 1936), for which he and Otto Loewi were awarded the Nobel Prize in Physiology or Medicine. In 1966 Katz reported the quantal and vesicular properties of ACh release (Katz, 1966). Since then, the cholinergic NMJ has proven a highly suitable experimental synapse model that has enabled detailed analysis of neurotransmission.

Cellular components

The NMJ comprises parts of the motor neuron, skeletal muscle fiber, and Schwann cell (Figure 1) (reviewed in Couteaux, 1973; Ogata, 1988; Engel, 1994). The motor axon, myelinated by Schwann cells, originates from the nerve cell body within the ventral horn of the spinal cord, and projects to the target muscle through the peripheral nerve. The terminal branches of the motor axon, which are each up to 100 μm long, can contact up to tens to hundreds of muscle fibers. The combination of a motor neuron and all of the muscle fibers it innervates is called a motor unit. The place of synaptic contact is at a site near to the middle of the muscle fiber, where invaginations in the postsynaptic membrane (primary clefts) harbor the axon terminals. The nerve terminal is covered by perisynaptic Schwann cells. They insulate the nerve terminal from the environment, support the long-term maintenance of the synapse and are capable to modulate neurotransmitter release. Schwann cells guide regeneration after injury as well and are play a crucial role during development (Sanes and Lichtman, 1999; reviewed by Feng and Ko, 2007).

The synaptic cleft between the nerve terminal and the postsynaptic membrane is about 50 nm wide and is lined with basal lamina, a thin layer of connective tissue that sheaths the muscle fiber. It consists of collagen IV, laminin, entactin, and heparan sulfate proteoglycans. The synaptic basal lamina also contains a collagen-tailed form of acetylcholinesterase, important for the degradation of ACh (Krejci et al., 1997).

The nerve terminal contains many mitochondria, microtubules and actin microfilaments. The most noticeable characteristic in electron microscopic images is the presence of many synaptic vesicles with a diameter of about 50 nm. Some of these are clustered at specialized sites at the presynaptic membrane, called 'active zones', appearing as electron-dense regions of the cytoplasm. Upon Ca^{2+} influx through voltage-gated Ca^{2+} channels ($Ca_v2.1$, also called P/Q type), these vesicles undergo exocytosis.

Localized opposite to the pre-synaptic membrane, the post-synaptic muscle membrane exhibits an eight-time increased surface area due to extensive folding

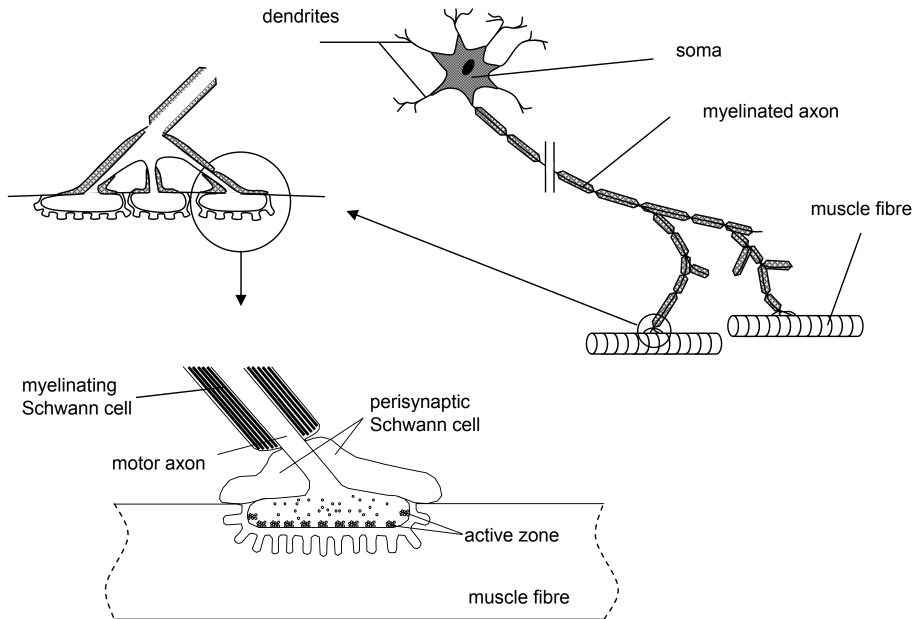

Figure 1. Motor neuron and NMJ
Schematic representation of the motor neuron, making synaptic contact with a number of muscle fibers, together forming the motor unit. The nerve endings are positioned in the primary folds of the muscle fiber membrane and are covered with perisynaptic Schwann cells. The nerve terminal contains synaptic vesicles filled with ACh. Upon arrival of a presynaptic action potential, the vesicles fuse with the presynaptic cell membrane at the active zones, thereby releasing ACh in the synaptic cleft. AChRs located on top of the secondary folds open after binding of ACh, permitting an ion current to flow which underlies a depolarization that activates voltage gated Na^+ channels located in the depths of the folds. This results in a muscle action potential that eventually induces contraction of the fiber. Modified from Plomp et al., 2003.

('secondary folds'). Voltage-gated Na^+ channels are localized in the depths of the folds. Several myonuclei with associated Golgi apparatus lie just beneath the post-synaptic membrane. On the surface of the secondary folds exposed to the synaptic cleft, large numbers of ACh-gated receptor-channels (AChRs) are present at high density (10000/μm^2). Upon binding of ACh, these channels open and cause a net inward ion current (see below). Extrasynaptically, the AChR density drops to 10/μm^2 (Fertuck and Salpeter, 1976).

The acetylcholine receptor

The endplate AChR is a ligand-gated ion channel of the nicotinic class. Binding of two ACh molecules results in a short opening of the pore, allowing influx of Na^+ and efflux of K^+ ions. This leads to a net inward electrical current. The discovery of α-bungarotoxin (α-BTx), a peptide component of the venom of the snake Bungarus multicinctus, enabled the purification and subsequent structural analysis of the AChR (reviewed in: Karlin, 2002). α-BTx is a highly specific irreversibly binding ligand that has been applied to extract AChRs from the Torpedo electric organ in large amounts. AChRs are heteromultimeric structures consisting of four different

polypeptides subunits: α, β, γ, and δ with a stoichiometry of α2βγδ. X-ray crystallography analysis shows a structure that resembles a chalice, with a large extracellular 'mouth' of 65 Å, and a smaller intracellular vestibule of 20 Å, connected by a 30 Å wide pore. The binding sites for ACh and α-BTx are located on the α-subunits. Embryonic mammalian NMJs possess AChRs with the same subunit composition as the Torpedo AChR; however, during the first postnatal week the γ-subunit is replaced by an ε-subunit. This yields a channel with a shorter opening duration and a larger electrical conductance (Mishina et al., 1986; Gu and Hall, 1988; Missias et al., 1996).

Function

The function of the NMJ is to transmit signals from the motor neuron to the muscle fiber, which is achieved by action potential-induced ACh release from the nerve terminal. ACh is synthesized from choline and acetyl coenzyme A (AcCoA) by the enzyme choline-acetyltransferase (ChAT) which transfers an acetyl group from AcCoA onto choline. In the synaptic cleft, ACh is rapidly hydrolyzed by acetylcholinesterase into choline and acetate, thereby terminating the ACh signal. Subsequently, choline is transported back into the nerve terminal and re-used for ACh synthesis. Cytoplasmic ACh is transported into synaptic vesicles by a vesicle specific proton pump (V-ATPase), packing about 10,000 molecules of ACh into one single vesicle.

Upon arrival of an action potential at the presynaptic terminal, voltage-gated Ca^{2+} channels open. These are $Ca_v2.1$ (P/Q-type) channels, also widely expressed in the CNS (Wheeler et al., 1995). In the PNS, $Ca_v2.1$ channels are mainly restricted to the NMJ (Uchitel et al., 1992). The opening of Ca^{2+} channels results in a short Ca^{2+} influx, causing a transient local elevation of the Ca^{2+} concentration, from 100 nM to 200-300 μM. The Ca^{2+} signal is transmitted to the exocytotic protein machinery and induces rapid fusion of vesicles from the readily-releasable pool (RRP) with the plasma membrane, thereby releasing ACh in the synaptic cleft. Vesicle fusion increases with $[Ca^{2+}]_n$, with n = 3-4, interpreted as three to four Ca^{2+} ions to work cooperatively in inducing fusion (reviewed by Meir et al., 1999).

ACh binds to the postsynaptic AChRs which in turn open and permit a net inward current of positive ions, the endplate current (EPC). The EPC underlies a depolarization, the endplate potential (EPP) (Fatt and Katz, 1951). Using electrophysiological techniques, EPPs can be measured in vitro (Figure 2, also see below). Next to the action-potential evoked EPPs, small depolarizations of ~1 mV can be observed at ~1/s, the miniature endplate potentials (MEPPs) (Fatt and Katz, 1952). MEPPs are the result of the spontaneous release of one single quantum of ACh. Typically, in the NMJ (depending on species, muscle-type and age), the quantal content of endplates (i.e., the number of vesicles that fuse upon a presynaptic action potential) varies between 25 and 100.

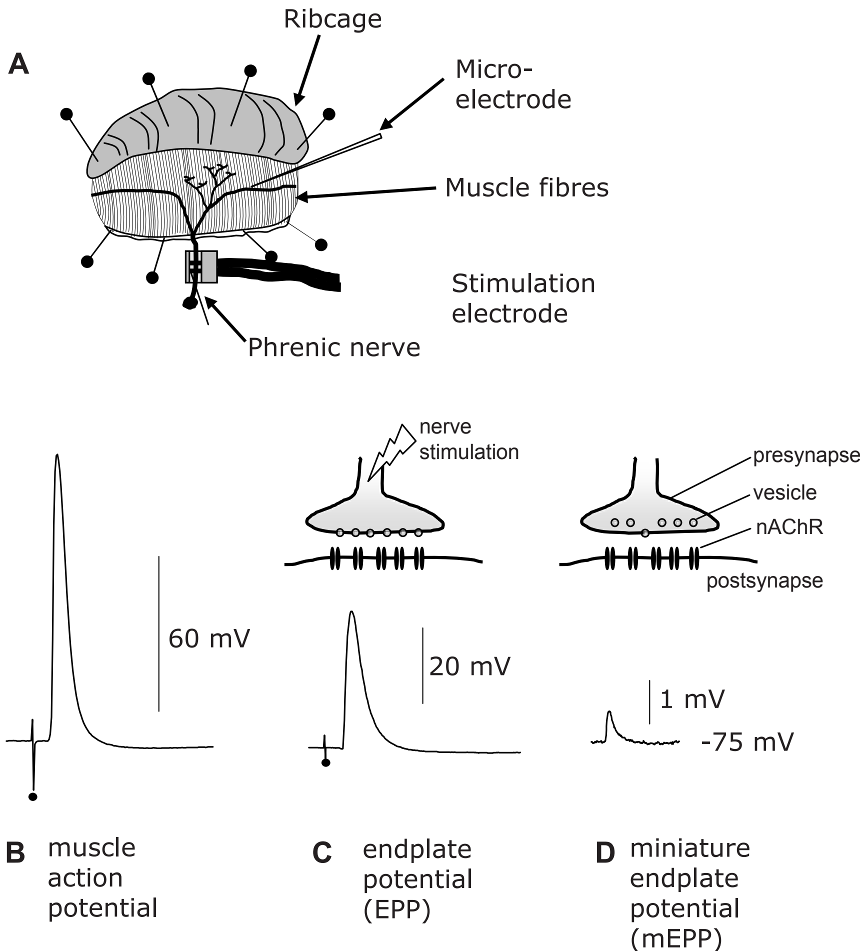

Figure 2. NMJ electrophysiology
Schematic representation of a hemidiaphragm, pinned out in a dish (A). The phrenic nerve has been placed on a bipolar stimulation electrode that delivers electrical pulses which trigger nerve-action potentials. Under microscopic inspection a muscle fiber is impaled with a microelectrode to monitor the membrane potential which typically is around -75 mV. Stimulation of the nerve (indicated with a black dot) will eventually result in a muscle action potential (B), which in most cases will disrupt the recording since it induces contraction of the muscle. µ-Conotoxin-GIIIB is used to block the muscle Na^+ channels in order to prevent muscle action potentials, enabling undisturbed measurement of the EPPs (C). Spontaneous release events can be monitored as MEPPs (D). The quantal content, which is the number of vesicles that have fused to give rise to an EPP is calculated by dividing the EPP amplitude by the MEPP amplitude. (Modified from (Kaja, 2007) with permission).

The precise function of MEPPs has not been established yet, but might involve regulation of local postsynaptic protein synthesis (Sutton et al., 2004). Otherwise, it may be an intrinsic property of the molecular machinery that mediates synaptic vesicle function (Lou et al., 2005). However, since spontaneous and evoked release seem to originate from distinct vesicle pools a functional relevance for spontaneous release cannot be excluded.

The EPP activates voltage-gated Na^+channels, which initiate a muscle action potential that leads to contraction of the muscle fiber. (Fatt and Katz, 1951). In

general, the EPP amplitude is several times higher than minimally required to excite the muscle fiber. This safety factor ensures that during prolonged, high-frequency activation of muscles, when the amount of transmitter released per nerve impulse declines substantially, transmission does not fail. (Wood and Slater, 2001).

In vitro electrophysiological analysis of ACh release at the NMJ

Several mouse nerve-muscle preparations can be used for studying NMJ electrophysiology. In this thesis, the diaphragm-phrenic nerve preparation was mainly used, as well as the soleus-tibialis nerve preparation. The diaphragm preparation has the advantage that it is a flat, thin muscle (typically 10-15 fibers thick) and that its fibers can be easily visualized and are well accessible by microelectrodes. In addition, NMJs are localized in a central band along both hemi-diaphragms that can be easily visually identified. A further advantage is the length of the phrenic nerve which can be dissected, so that it can be placed over a bipolar electrode for stimulation (Figure 2A). The diaphragm is a mixture of both slow- and fast-switch fibers. The soleus muscle is a technically somewhat more demanding preparation, as the NMJ region is not very well identifiable and nerve stimulation requires a suction electrode configuration. Soleus muscles consist of slow-twitch fibers. After dissection, the muscles are pinned on a silicone rubber-coated dish containing a physiological Ringer's solution.

A glass micro electrode (1 μm tip diameter), coupled to an amplifier, is impaled in the muscle cell near the endplate-region using a micromanipulator. The membrane potential is then measured, which is in rest around -75 mV. MEPPs can be recorded; typical values for diaphragm are 1 mV, occurring at a frequency of 1 s^{-1}. Using a stimulus generator, the nerve can be electrically stimulated and the ensuing EPPs can be recorded. Because the measured resting membrane potential varies between the multiple muscle fibers sampled, amplitudes at each NMJ are normalized to -75 mV.

Measures have to be taken to prevent muscle action potentials, since these obscure the EPP and cause contraction which in general will disrupt the recording. Several methods exist to prevent muscle action potentials; the most straight-forward of these is selectively blocking the muscle Na^+ current by μ-conotoxin-GIIIB, a marine snail venom component. Other methods involve reducing the EPP amplitude to sub-threshold values for Na^+ channel activation by blocking part of the AChRs using the reversible antagonist d-tubocurarine. The drawback of this method is that MEPPs will become too small to be measured.

From the EPP and MEPP values recorded, the quantal content at an endplate can be calculated by dividing the EPP amplitude by the MEPP amplitude. Beforehand,

the EPP amplitude needs to be corrected for non-linear summation (McLachlan and Martin, 1981).

Some of the experiments described in this thesis have been carried out on embryonic or one day old mice. In addition to a slightly more complicated dissection, it appeared that muscle action potentials could not be blocked by μ-conotoxin-GIIIB. In these experiments, a depolarization-induced inactivation of the Na^+ channels was used to prevent muscle action potentials. To this end, diaphragm muscle fibers were shortened by cutting away some length from the central tendon side until depolarization of the muscle fibers to about -40 mV was reached, preventing the triggering of action potentials by EPPs.

Myasthenia gravis

Clinical symptoms

MG is a prototype for both synaptic and autoimmune disorders, with a prevalence of around 20-500 per million (reviewed by Lindstrom, 2000; Vincent et al., 2001). The disorder is characterized by a painless, fatigable weakness. At onset, patients often have ptosis (eyelid drooping) and diplopia (double-vision) arising from levator palpebrae and extraocular muscle weakness. Typically within one year, 75% of the patients will develop bulbar weakness (for example, facial weakness, difficult chewing and defective articulation) and/or extremity weakness. Weakness can remain localized to one group of muscles for many years (commonly in the eye-muscles, termed 'ocular myasthenia') or spread to affect other skeletal muscles (generalized MG). Myasthenic crises (life threatening episodes of respiratory or bulbar paralysis) may occur.

MG is often accompanied by thymic abnormalities, and around 10% of patients suffer from a lymphoepithelial thymoma (Oosterhuis, 1989).

Histological analysis of intercostal biopsies from myasthenic patients show a reduction or disappearance of functional folds, with widened secondary folds and synaptic cleft (Vincent, 1987).

Mechanism

The majority (85%) of patients with MG is seropositive for antibodies against the nicotinic AChR. The titers of antibodies are highly variable amongst patients, and do not correlate well with severity of the clinical symptoms between individuals. However, individual patient titers correlate well with clinical scores after plasma exchange, thymectomy and/or immunosuppressive treatment. The remaining 15% of the patients is classified as seronegative MG. In a subset of this group antibodies against muscle specific kinase (MuSK) have been found. The proportion of seronegative MG patients with antibodies against MuSK seems to vary (60-70% in sera

from Oxford (Hoch et al., 2001) and Italy, 30-40% in sera from the USA and Japan, and none has yet been identified in Norway (reviewed in: Vincent and Leite, 2005).

The autoantibodies decrease the ACh sensitivity of the NMJ, thereby reducing the safety-factor of neuromuscular transmission. In healthy NMJs, the EPP amplitudes decrease during repetitive stimulation (i.e. during voluntary muscle contraction) which is probably the result from depletion of the vesicle pool in the presynaptic terminal in combination with the behaviour of $Ca_v2.1$ channels during high frequency stimulation. However, due to the safety-factor of the NMJ, the EPPs will remain supra-threshold and will all elicit muscle action potentials. However, the reduced safety factor of MG NMJs results in sub-threshold EPPs during repetitive stimulation which results in the observed (fatigable) weakness of patients.

There are three mechanisms by which the autoantibodies to the AChR lead to decreased ACh sensitivity at the NMJ: 1) complement-mediated damage of the post-synaptic membrane by antibodies, 2) increased internalization due to cross-linking by antibodies and 3) AChR block by direct binding of antibodies (reviewed by: Boonyapisit et al., 1999). Complement-mediated lysis is by far the most important effect.

Depending on the type of MG and the severity of the symptoms several treatments are given. These include the administration of acetylcholinesterase inhibitors (in order to prolong the life-time of ACh in the synaptic cleft), thymectomy, plasma-exchange and immune-suppressive therapy.

Clinical and in vitro electrophysiology

Electromyographical methods are used in the diagnosis of MG (reviewed in Meriggioli and Sanders, 2004). The two tests that are used are repetitive nerve stimulation (RNS) and single-fiber electromyography (SFEMG). In RNS the peripheral nerve is stimulated supramaximally and the compound muscle action potential (CMAP) is recorded with surface electrodes. In healthy muscles, the amplitudes of the CMAPs will remain constant during the repetitive stimulation. In MG muscles however, the CMAP amplitude decreases during the train of stimuli because an increasing number of fibers no longer contracts due to EPPs that become sub-threshold for action potential generation. In SFEMG, a needle is used to record from an individual muscle fiber during contraction of a muscle (voluntary or stimulated). There is a small variation in the delay between the stimulation and the recorded muscle action potential between the successive stimuli. This 'jitter' phenomenon is caused by the variation in the time it takes for the EPP to reach the threshold and the initiation of the muscle action potential. Increased jitter is reflecting a defect in neuromuscular transmission, EPPs being peri-threshold. Sometimes an EPP will not be able to generate a muscle action potential at all, which is termed a 'blocking'.

In vitro electrophysiological recording from muscle biopsies from MG patients showed a decreased MEPP amplitude (Elmqvist et al., 1964), indicating a decreased postsynaptic sensitivity for ACh. EPPs, measured at low frequency stimulation, showed a decreased amplitude, which is however higher than would be expected based on the MEPP amplitude reduction. This is caused by an increase in the quantal content (Cull-Candy et al., 1980; Plomp et al., 1995), resulting from a homeostatic presynaptic response of the NMJ in attempt to maintain successful transmission (see below). During high frequency stimulation of the nerve, EPPs show increased rundown of amplitude, compared to normal.

Experimental models of myasthenia gravis

Several animal models of MG have been developed in order to study the pathophysiological mechanisms and possible treatments of this disease.

The first model of MG comprised injections of rabbits with AChR purified from the Torpedo electrical organ, which caused paralytic symptoms. This experimental auto-immune myasthenia gravis (EAMG) model provided much information about the pathophysiological mechanisms of MG but the condition of the animals proved to be difficult to control. After an initial phase with mild muscle weakness a more generalized MG follows, including breathing problems which ultimately lead to death of the animals.

A more controllable model is toxin-induced MG (TIMG), in which the decreased ACh-sensitivity is achieved by repeated intraperitoneal injections with low doses of α-BTx (Figure 3). Initially developed for rats (Molenaar et al., 1991), it has been changed and re-evaluated for the use in mice.

Homeostatic upregulation of neurotransmitter release at (neuromuscular) synapses

As outlined in the previous section, myasthenic synapses aim to compensate for the decreased sensitivity of the postsynaptic cell to ACh by releasing more ACh upon nerve stimulation. This compensatory increase of quantal content has been found in NMJs from MG patient muscle biopsies (Molenaar et al., 1979; Cull-Candy et al., 1980), and has been confirmed by recordings in NMJs from animal models for MG (Molenaar et al., 1979; Cull-Candy et al., 1980; Takamori et al., 1984; Molenaar et al., 1991; Plomp et al., 1992; Plomp et al., 1995). The increase in quantal content is correlated with the reduction of the MEPP amplitude, indicating that regulation takes place at individual endplate level (Plomp et al., 1992; Plomp et al., 1995).

The increase of neurotransmitter release in the myasthenic NMJ suggests that the neural activity in the NMJ is under homeostatic control (Davis and Bezprozvanny, 2001; Burrone and Murthy, 2003). Homeostatic regulation of transmitter release in response to decreased postsynaptic sensitivity in the NMJ has been described in

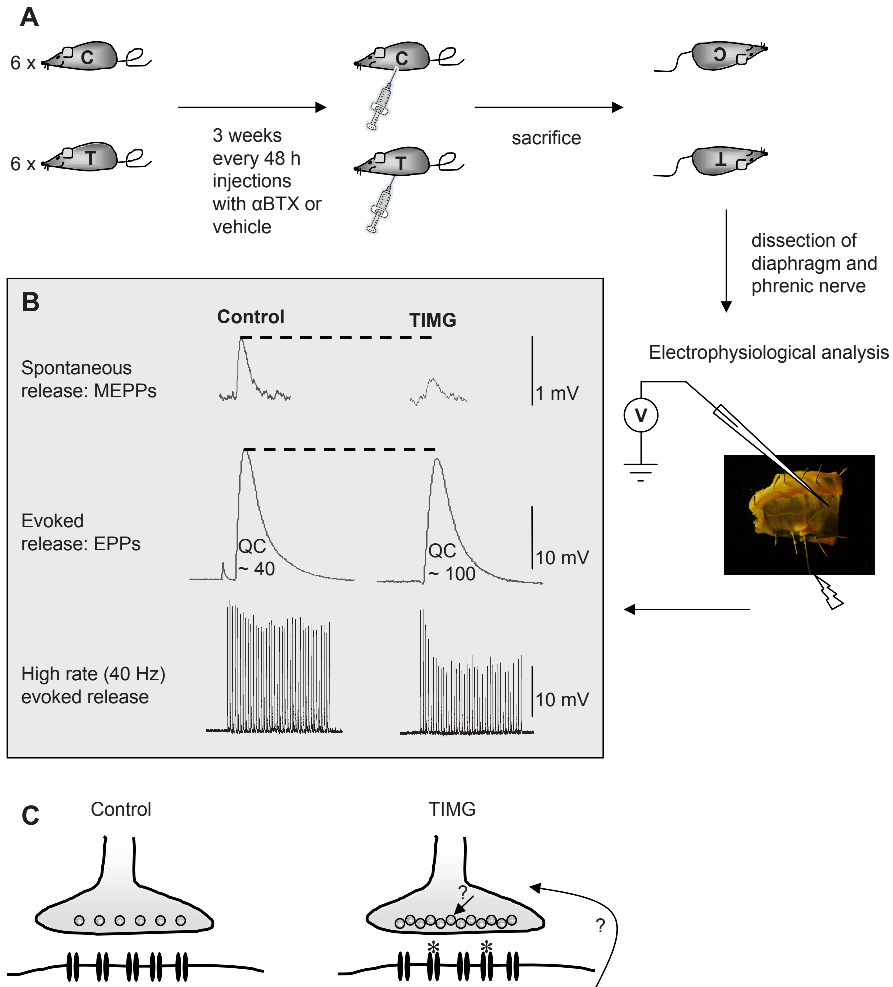

Figure 3. Toxin-induced myasthenia gravis
Toxin-induced myasthenia gravis (TIMG) is a non-immunogenic model of MG, where reduced ACh sensitivity of the NMJ is induced by injection of the nAChR-blocker α-BTx into mice.
(A) For 3 weeks, mice receive every 48 h an injection with either a 0.8 μg dose α-BTx ('T') or control vehicle ('C').
(B) Reduced MEPP amplitudes in TIMG reflect the reduced ACh sensitivity due to block of part of the AChRs by α-BTx at the NMJ. Typically, the EPP amplitudes in TIMG NMJs are only slightly reduced, hence the quantal content of these NMJs has been increased to compensate for the reduced ACh sensitivity. High rate (40 Hz) evoked release in TIMG NMJs typically shows increased rundown. (C) Schematic representations of control and TIMG NMJs (star indicates α-BTx.). Arrows indicate unknown pathways that are being employed to increase vesicle fusion in response to AChR blockade.

additional models. For example, in mice heterozygous knock-out for the Ig-containing isoform of neuregulin the AChR density was reduced. This was compensated for by increased quantal content (Sandrock, Jr. et al., 1997). A similar phenomenon was seen in studies at the glutamatergic *Drosophila* NMJ. In one of these studies mutations were induced in the glutamate receptor subunit GluRIIA (Petersen et al., 1997). Expression of a dominant negative form of GluRIIA resulted in the decrease

of the response to single quantum. However, the response to nerve stimulation was the same due to an increase in quantal content. In another study a constitutively active catalytic subunit of protein kinase A (PKA) was expressed (Davis et al., 1998) to achieve decreased postsynaptic sensitivity. In a third *Drosophila* study the excitability of the postsynaptic muscle was decreased by postsynaptic expression of the $K_{ir}2.1$ K^+ channel (Paradis et al., 2001). In all these cases the reduced postsynaptic sensitivity for the transmitter was (partly) counteracted by a compensatory increase in presynaptic transmitter release.

Homeostatic control of presynaptic release requires retrograde signaling to provide the presynapse with feedback on the current state of neurotransmission. Many candidate retrograde messengers have been proposed, including soluble factors like brain-derived neurotrophic factor (BDNF), neurotrophins, nitric oxide, and endocannabinoids (Fitzsimonds and Poo, 1998; Tao and Poo, 2001). Especially BDNF has been given much attention (Lohof et al., 1993; Lessmann et al., 1994; Berninger and Poo, 1996; Lessmann, 1998; Schinder et al., 2000; Tyler et al., 2002). Magby and colleagues described the action of BDNF as a retrograde messenger in cultured hippocampal neurons (Magby et al., 2006). It was proven by pharmacological means that BDNF was released from postsynaptic neurons upon depolarization, and that this had a direct enhancing effect on the spontaneous transmitter release of the presynaptic cell. This process was dependent on activation of presynaptic tyrosine kinase B (trk-B) and the postsynaptic intracellular Ca^{2+} concentration.

Direct cell-cell contact by transsynaptic protein complexes may be employed as well in retrograde signaling (Davis and Bezprozvanny, 2001; Dean and Dresbach, 2006; Futai et al., 2007). An example of these trans-synaptic protein complexes that mediate presynaptic release in the CNS is a neuroligin-neurexin complex, formed by presynaptic Neurexin-β and the postsynaptic complex PSD-95-Neuroligin. Futai and colleagues described an experiment performed in cultured hippocampal neurons where the levels of postsynaptic PSD-95 and neuroligin were found to affect presynaptic release. This mechanism was dependent on the presence of presynaptic β-neurexin (Dean and Dresbach, 2006; Futai et al., 2007).

Studies in *Drosophila* have revealed the bone morphogenic protein (BMP) glass bottom boat (Gbb) as a retrograde signal in homeostatic synaptic plasticity. As mentioned above, expression of a dominant negative of GluRIIA induced a compensational increase of transmitter release, but this was not the case in mutants lacking the BMP receptor wishful thinking (Wit) (Haghighi et al., 2003). Gbb is a good candidate for the retrograde signal because it is expressed by developing muscle fibers, and Gbb deletion mutants have a similar phenotype as Wit deletion mutants (Keshishian and Kim, 2004). However, more recent evidence seems to contradicts this because both post- and presynaptic expression of Gbb restores synaptic ho-

meostatis in Gbb deletion mutants (Goold and Davis, 2007). Thus, the identity of the retrograde signal remains unknown.

Another *Drosophila* study on a specific motoneuron-interneuron synapse describes a Gbb-Wit indendent example of synaptic homeostatis. Deleting Dp186, one of the *Drosophila* isoforms of Dystrophin increases evoked presynaptic release. Mutants can be restored to wild-type levels by postsynaptic (but not presynaptic) expression of Dp186 (Fradkin et al., 2008), indicating that the protein is involved in the retrograde signaling pathway. Interestingly, another Dystrophin isoform (DLP2) has shown to be involved in a similar manner in synaptic homeostasis in the NMJ (van der Plas et al., 2006). Recently, yet another protein, Dysbindin, was found to play a role in homeostatic modulation of neurotransmission (Dickman and Davis, 2009). Dysbindin is necessary on the presynaptic side in synaptic homeostasis. It colocalizes with synaptic vesicle proteins, indicating that it functions at or near the synaptic vesicle pool. Dysbindin has been linked to schizophrenia in humans, suggesting that a defect in synaptic homeostasis is possibly contributing to this disease. However, this remains to be investigated.

Synaptic exocytosis

Exocytosis is defined as the process by which molecules are secreted from eukaryotic cells through fusion of membrane-bound vesicles with the plasma membrane. The most extensively studied example of exocytosis is chemical synaptic transmission, used intensively by cells in the nervous system to transduce electrical signals. Upon arrival of an electrical signal in the presynaptic terminal, fusion of vesicles takes place. The released transmitter substance binds to receptor molecules on the postsynaptic cell, which in turn generates an electrical signal that travels onwards.

Vesicle pools and life cycle

Synaptic vesicles in the nerve terminal are organized in different pools, depending on their level of fusion-readiness (Rizzoli and Betz, 2004). Vesicles in the readily releasable pool (RRP) are primed for fusion and await the Ca^{2+} trigger, in contrast to vesicles in the reserve pool (RP). Vesicles in this pool have to undergo several maturation steps in order to 'prime' them to make them readily releasable. These pools together are called 'recycling pool'. There is a third pool, the 'resting pool' of vesicles. The latter pool most likely provides the vesicles that undergo spontaneous release, while the RRP is responsible for regulated release (Fredj and Burrone, 2009)

Vesicles that take part in regulated exocytosis have a life cycle, starting with the synthesis of the lipids and membrane proteins in the endoplasmatic reticulum and modification in the Golgi apparatus. The vesicle then is translocated from the soma to the nerve terminal, where neurotransmitter molecules are transported in the lu-

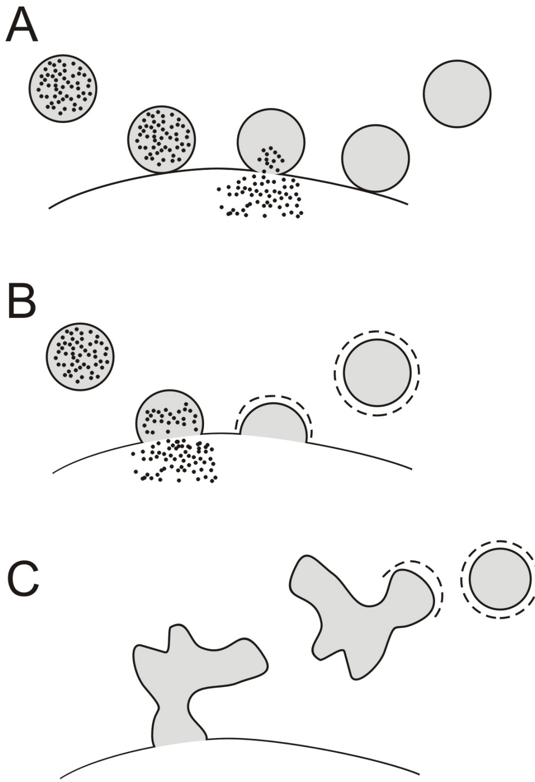

Figure 4. Endocytotic pathways
Three different pathways have been proposed for endocytosis as depicted in this figure. (A) "Kiss and run", where the vesicle fuses, is emptied and closes again, and refilling takes place after it has been relocated from the site of fusion. (B) The vesicle fuses completely with the membrane, loosing its identity. The membrane is retrieved by clathrin coated pits (Clathrin is depicted by black bars) (C) A larger amount of membrane can be retrieved in bulk endocytosis. An endosomal structure is formed, from which vesicles can bud.

reserve pool of vesicles, or it will be directed to the active zone. At the active zone the vesicle docks at the plasma membrane and undergoes priming steps, making it ready ('competent') for Ca^{2+}-triggered fusion-pore opening.

Three endocytotic modes have been proposed (Figure 4) (reviewed by Smith et al., 2008). One mode is the 'kiss-and-run' pathway, where vesicles are retrieved before full collapse has occurred, maintaining the vesicle identity. Another mode is full-collapse fusion, where complete fusion of the vesicle with the membrane takes place, the vesicle membrane has become part of the cell membrane. Retrieval of the membrane occurs via clathrin coated pits. Alternatively, retrieval can take place via 'bulk endocytosis' where an endosomal structure is formed. In this case new vesicles are formed by budding from the endosomal structure. Most likely, these three modes coexist in synapses.

Neuro-exocytotic proteins

A large number of different proteins is implicated in the regulation of the different stages of the synaptic vesicle life cycle. Whereas some of them are essential, others may have auxiliary or regulatory functions. The basic mechanism of exocytosis is conserved from yeast to man and in different cell-types. Although homologous proteins acting in specific steps in exocytosis have been identified across many species,

it has become clear that there is a wide variety of differences in the specific steps of exocytosis, and homologous proteins may have distinct roles in different cell types or organisms. Therefore, many conflicting reports on the precise roles of some of the exocytotic proteins exist. To discuss the detailed roles of all existing neuro-exocytotic proteins is beyond the scope of this thesis Introduction. I will limit myself to a description of the soluble N-ethylmaleimide-sensitive factor attachment protein receptors proteins (SNAREs) (Figure 5), which are believed to be the key-role players of fusion and, in addition, the proteins studied in the experimental chapters of this thesis (Figure 6).

SNAREs

At present, it is widely accepted that SNAREs are the key players in the final stages of docking and subsequent fusion of synaptic vesicles (Jahn and Sudhof, 1999; Chen and Scheller, 2001; Rizo and Sudhof, 2002; Jahn et al., 2003). They are small proteins (10-35 kDa), characterized by the SNARE motif, which comprises of a stretch of 60 to 70 amino acids, often localized next to a C-terminal transmembrane anchor. Most SNARE proteins contain one SNARE motif. However, the soluble NSF attachment proteins (SNAPs) SNAP-25, SNAP-29 and SNAP-23 contain two of them. SNAREs can be functionally divided into vesicle-associated (v-), and target-associated (t-) SNAREs (Sollner et al., 1993). However, as these functional categories do not apply to all types of fusion reactions (e.g. fusion of yeast vacuoles), a categorization based on a single key residue in the SNARE motif, being either arginine (R) or glutamine (Q) was developed (see below).

Unstructured in solution, SNAREs can assemble into 'SNARE-complexes', which are formed by four SNARE motifs adopting an α-helical configuration in the center of which there are four conserved, interacting amino acids (three glutamines, one argine). Based on this, a new classification has been made, Qa-SNAREs (or syntaxins), Qb-, and Qc- SNAREs (homologs of the N- and C-terminal SNARE motif, respectively, of SNAP-25), and R-SNAREs (vesicle-associated membrane proteins, VAMPs, also called synaptobrevins) (Fasshauer et al., 1998). The four α-helices zip together from the N-terminal end of the SNARE motifs toward the C-terminal membrane anchors, thereby pulling the membranes close together (Hanson et al., 1997; Lin and Scheller, 1997).

It is generally accepted that SNAREs provide the energy necessary to overcome the energy barrier for membrane fusion through their assembly into SNARE complexes. These are extraordinarily stable, indicating that a lot of energy was released upon assembly (Fasshauer et al., 2002).

In neuronal exocytosis, the t-SNARES syntaxin 1a (Qa) and SNAP-25 (Qb and Qc) are predominantly located on the plasma membrane, whereas the v-SNARE VAMP/synaptobrevin (R) is located on the synaptic vesicle (Figure 5). If the com-

plex is located on both the vesicle and plasma-membrane, it is called a trans-SNARE complex. After fusion however, the complex is located only on the plasma membrane, in 'cis-configuration', and needs to be disassembled. Disassembly is achieved by action of the ATP-ase N-ethylmaleimide-sensitive fusion protein (NSF). In order to recruit NSF, binding of the co-factors SNAPs is required. ATP hydrolysis by NSF leads to disassembly of the complex (Sollner et al., 1993). After this, v-SNAREs are recycled to synaptic vesicles, while the t-SNAREs are re-organized for new rounds of docking and fusion events (Figure 5)

Using in vitro assays of liposome fusions, it has been shown that SNARE proteins alone are sufficient for the actual fusion of membranes, although the kinetics of this reaction are very slow (Weber et al., 1998). However, several observations argue against their exclusive role in membrane fusion. First, deletion of the R-SNARE synaptobrevin in mice does not completely abolish synaptic exocytosis (10% remains) in mice (Schoch et al., 2001). Similar results were obtained from mice lacking the t-SNARE SNAP-25 (Washbourne et al., 2002). Second, deletion of the non-SNARE protein Munc18-1 abolished exocytosis completely (Verhage et al., 2000). This shows that SNARE proteins alone are not sufficient for fusion. Thus, although being the core proteins of membrane fusion, other factors in addition to SNAREs are required for exocytosis in vivo.

Synaptic communication of neuronal cells is a very delicate and precise process, which is highly tuned in time and space. Precise control of synaptic exocytosis has evolved, in which many proteins play a role in the several aspects of exocytosis. Below, a subset of these is discussed, with relevance to the experimental studies of this thesis. For more information see the following reviews: (Jahn and Sudhof, 1999; Chen and Scheller, 2001; Rizo and Sudhof, 2002; Jahn et al., 2003; Sudhof, 2004; Sudhof and Rothman, 2009).

Munc18

Munc18-1 is a member of the Sec1/Munc18 protein family, referred to as SM proteins (Toonen and Verhage, 2003), consisting of highly conserved cytosolic proteins of ~60 to 80 kD. Deletion of SM proteins generally leads to an impairment of vesicle trafficking and fusion, and is lethal in most organisms (reviewed in Toonen and Verhage, 2003). Although all SM proteins have shown to be important in vesicle trafficking and fusion, it appeared difficult to pinpoint their exact roles (Weimer and Jorgensen, 2003; Toonen and Verhage, 2003; Weimer and Richmond, 2005).

Munc18-1 was first discovered as a binding-partner of syntaxin-1 (Hata et al., 1993) and was found to be the mammalian homologue of the *C. elegans* protein Unc18, deletion of which gives an uncoordinated movement phenotype (Hosono et al., 1992). Deletion of Munc18-1 in mice resulted in a lethal phenotype at birth and a complete blockade of synaptic vesicle fusion (Verhage et al., 2000). Munc18-1 is

one of the seven SM proteins discovered in mammals and is exclusively expressed in neurons (Toonen and Verhage, 2003).

SM proteins are arch-shaped molecules consisting of three domains that form a central V-shaped cleft (Bracher et al., 2000; Misura et al., 2000). The interaction with syntaxin-1 is a common feature of SM proteins (reviewed in Toonen and Verhage, 2003).

Munc18-1 binds to syntaxin in two different modes, namely to the 'open' and the 'closed' form of syntaxin. In the 'closed' form of syntaxin, the N-terminal Habc domain of syntaxin folds back on the C-terminal SNARE motif and blocks SNARE complex assembly (Dulubova et al., 1999). Munc18-1's arch shaped form clasps this 'closed' conformation of syntaxin (Misura et al., 2000). Munc18-1 can also bind via its N-terminal to an N-terminal sequence of syntaxin, leaving its arch shaped cavity

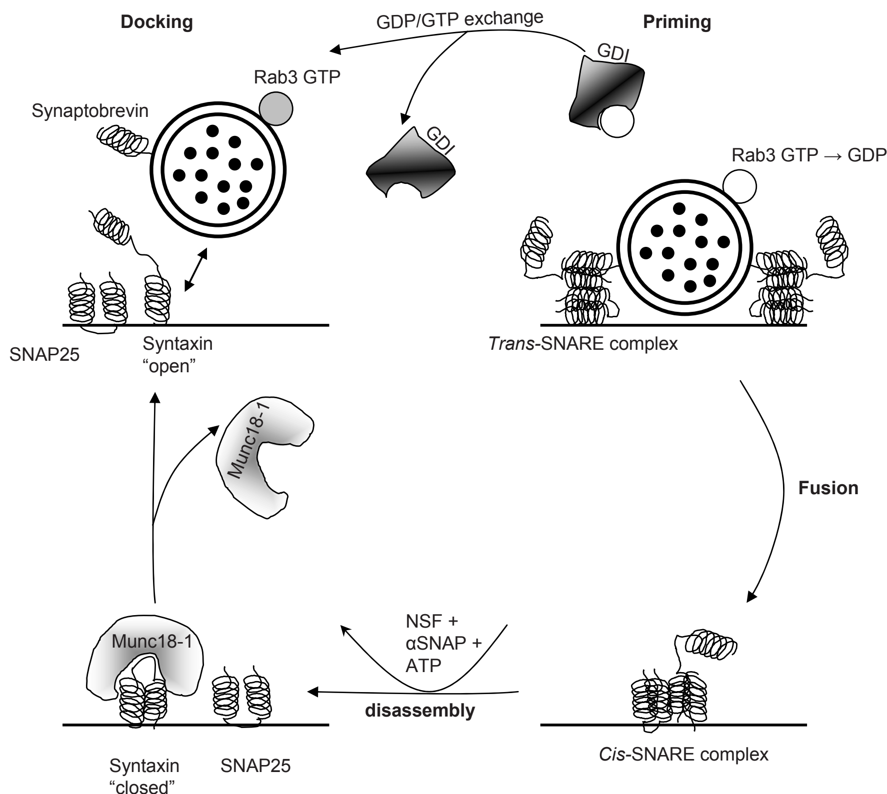

Figure 5. Vesicle fusion protein machinery
Conformational cycle of SNARE proteins. Syntaxin in 'open' state is able to form a trans SNARE complex with SNAP25 and synaptobrevin, releasing enough energy to induce membrane fusion. After fusion, αSNAP and NSF bind, and after ATP hydrolysis induce disassembly of the cis SNARE complex. Munc18-1 binds to the 'closed' state of syntaxin and needs to unbind to induce the open state. Not depicted here is the relocation of synaptobrevin, most likely upon retrieval of the vesicle membrane. The top panels include a schematic representation of the 'Rab3A' cycle, from the vesicle-bound GTP form, which is after ATP hydrolysis relocated by GTP-dissociation inhibiting protein (GDI)

open. This cavity then can be used to bind the assembling four-helical structure of the SNARE complex (see Sudhof and Rothman, 2009).

There has been a lot of debate about SM proteins being either positive or negative regulators of release. Genetic deletion of SM proteins results in a strong impairment or abolishment of release, suggesting a positive role of SM proteins. However, data on increasing SM protein levels (overexpression, injection) has led to conflicting results, suggesting either a positive or a negative role for the SM protein in exocytosis (Schulze et al., 1994; Dresbach et al., 1998; Wu et al., 1998; Voets et al., 2001). The most likely explanation is that the dual mode of interaction of the SM proteins and their cognate SNARE proteins represents two roles of the SM proteins. In membrane fusion, SM proteins have positive role (Shen et al., 2007) via the binding to the 'open' form of syntaxin and providing selectivity as well since binding only occurs to the cognate syntaxin. In addition, SM proteins can have a chaperone role to protect syntaxin during intercellular transport via binding to the 'closed' form of syntaxin (Medine et al., 2007).

Munc13

Unc13 was discovered in a classical genetic screen for *C. elegans* aiming at identifying mutant genes responsible for 'uncoordinated movements' (Brenner, 1974). It has one *Drosophila* homologue, Dunc13 (Aravamudan et al., 1999). Three Munc13 isoforms function in synaptic exocytosis (Munc13-1, bMunc13-2/ubMunc13-2 (splice variants of the Munc13-2 gene), and Munc13-3). Two other, ubiquitously expressed isoforms, presumably act in non-synaptic forms of exocytosis (Munc13-4 (Feldmann et al., 2003) and BAP-3 (Shiratsuchi et al., 1998)).

Munc13 proteins are large multidomain proteins with variable N-terminal sequences, but conserved central and C-terminal domains: a C2B (Ca^{2+} binding) domain, a large Munc13-homology region (the MUN domain) and a Ca^{2+}-independent C2C domain.

Deletion of Munc13 proteins in *C. elegans*, *Drosophila*, and mice showed severely reduced evoked and spontaneous release, but a normal number of docked vesicles (Aravamudan et al., 1999; Augustin et al., 1999; Richmond et al., 1999). The readily releasable pool of vesicles is strongly reduced, indicating that Munc13 acts after docking but prior to acquisition of 'fusion competence', called priming.

Munc13 interacts with syntaxin via its MUN domain, overexpression of which rescues synaptic transmission in Munc13 deletion (Stevens et al., 2005). Unc13 binds syntaxin at the same binding site as Unc18, and is even able to replace Unc18 (Sassa et al., 1999).

Munc13 and Munc18 have overlapping binding sites in the regulatory Habc domain of syntaxin. Munc13 could potentially displace Munc18 when it is clamping syntaxin in the 'closed' conformation to enable transition to the 'open' conforma-

tion, thereby enabling SNARE complex formation. Correspondingly, expression of a constitutively 'open' mutant of syntaxin (but not wild-type) rescued the release properties of a *C. elegans* Unc13 mutant (Richmond et al., 2001).

Munc13 participates in a tripartite complex with Rab3 and RIM, presumably to bring vesicles to the fusion machinery (Dulubova et al., 2005). Recent data suggests that this complex might be much more complex, also involving big presynaptic active zone proteins like Bassoon, Acyonin/Piccolo and CAST. The N-terminus of Munc13 seems to be site where all binding converges (Wang et al., 2009).

Next to being involved in basic release of neurotransmission, Munc13 has been shown to play a role in plasticity as well. The N terminal C1 domain is involved, which binds phorbol ester [4ß-phorbol-12, 13-dibutyrate (PDBu)] and diacyglycerol (DAG). This interaction is crucial for short term plasticity in the mouse hippocampus (Rhee et al., 2002). In addition, recently it was shown that the C2B domain of Munc13 functions as a Ca^{2+} regulator of short-term synaptic plasticity as well (Shin et al., 2010).

a-Neurexin

Neurexin1α was originally discovered as a neuronal cell-surface protein that binds the neurotoxin α-latrotoxin (Ushkaryov et al., 1992; Petrenko et al., 1993). The latter stimulates synaptic vesicle exocytosis and induces massive neurotransmitter release (reviewed in: Sudhof, 2001). Neurexin1α is a member of the neurexin protein family, which in mammals is encoded by three large genes (neurexin1, -2, and -3) (Tabuchi and Sudhof, 2002). Each of these genes contains a separate promoter for α- and β-neurexins. Neurexin genes are subject to intensive alternative splicing, potentially giving rise to thousands of isoforms (Missler and Sudhof, 1998).

α-Neurexins are transmembrane proteins, containing an N-terminal signal peptide followed by a three times repeated domain. Intracellularly, α-neurexins bind structural proteins with PDZ domains, Ca^{2+}/calmodulin-dependent serine protein kinase (CASK), Munc interacting protein (Mint) and the SNARE synaptotagmin. Extracellularly, α-neurexins bind to the postsynaptic cell-adhesion proteins dystroglycan (Missler and Sudhof, 1998; Sugita et al., 2001), neurexophilin (Missler et al., 1998) and neuroligin (Boucard et al., 2005).

Based on their structure and binding partners, neurexins have been suggested to function as cell-adhesion molecules that play an active role in the functional organization of the presynaptic machinery (Missler et al., 1998; Missler and Sudhof, 1998; Sudhof, 2001; Tabuchi and Sudhof, 2002; Missler et al., 2003). Deletion of α-neurexins in mice, however, showed morphologically normal synapses in brain. Interestingly, mutants lacking all three isoforms of α-neurexin display reduced synaptic N-type Ca^{2+} channel function, but unchanged numbers of cell-surface Ca^{2+}

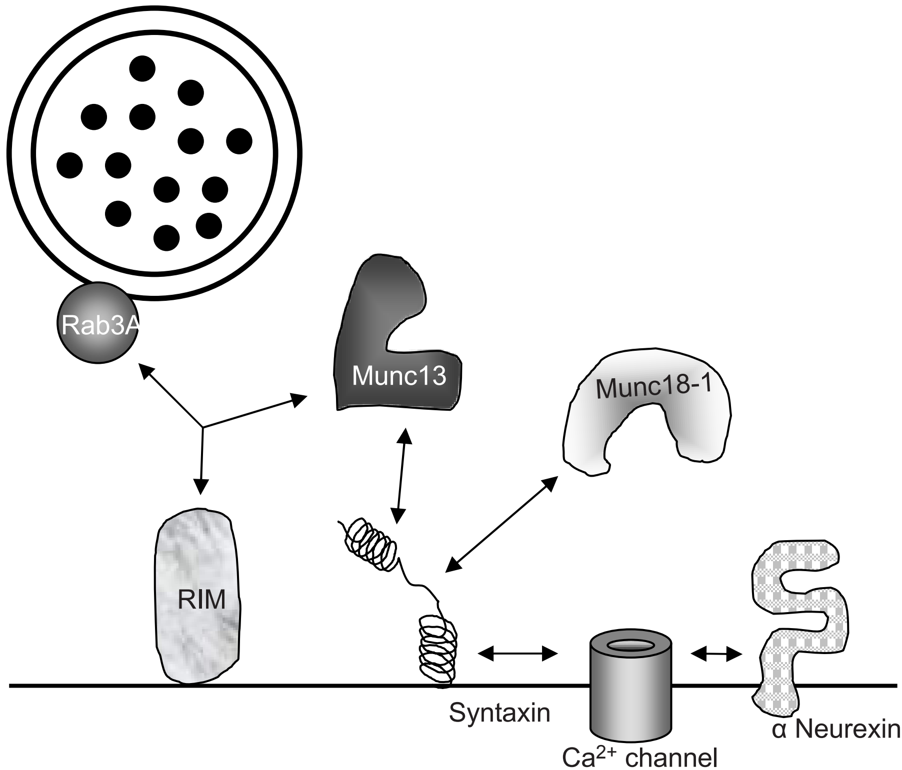

Figure 6. Exocytotic proteins studied in this thesis
Depicted here are the proteins that have been studied in this thesis (indicated with an asterisk), along with some key-role players in the process of exocytosis. Arrows indicate interactions, see main text for further details.

channels, suggesting that α-neurexins play a role in functionally coupling Ca^{2+} channels to the exocytotic machinery.

As mentioned above, β-neurexin has been implicated in a transsynaptic retrograde signaling pathway with neuroligin (Futai et al., 2007). α-Neurexins potentially could act in a similar pathway with a specific splice variant of neuroligin (Boucard et al., 2005).

Rab3A

Rab3A, B, C and D are small GTP-binding proteins that are involved in regulating membrane traffic (reviewed by Darchen and Goud, 2000). Rab3A, the most abundant brain isoform (Geppert et al., 1994), is associated with synaptic vesicles, but dissociates upon GTP-to-GDP hydrolysis during, or shortly after the fusion event (Fischer von Mollard et al., 1991; Star et al., 2005). Rab3A is therefore alternating between a GTP- and a GDP-bound form, which may function as a molecular switch regulating interaction with binding partners.

Rab3A binds to rabphilin (Shirataki et al., 1993), Rab-interacting molecule (RIM) (Wang et al., 1997), and synapsin-I (Giovedi et al., 2004). Deleting Rab3A in mice

only leads to a mild synaptic phenotype that includes altered short-term synaptic plasticity, and the absence of PKA-dependent long-term potentiation (Castillo et al., 1997; Geppert et al., 1997), suggesting a modulatory role for Rab3A. Mice that lack Rab3A, B, C, and D (quadruple knock-outs) are not viable, while the individual knock-outs display no or only a mild phenotype, indicating that Rab3 proteins are indispensable but individually are functionally redundant (Schluter et al., 2004). Analysis of cultured hippocampal neurons from these quadruple knock-out mice showed impaired evoked neurotransmitter release, whilst leaving spontaneous release unchanged (Schluter et al., 2004). The *C. elegans* Rab3 homologue interacts with the RIM homologue and this interaction is likely to play a role in placing the vesicle in the proximity of the presynaptic calcium channels (Gracheva et al., 2008).

RIM1a

Rab interacting molecule-1α (RIM1α) is located presynaptically at the active zone and plays an important role in neurotransmitter release (Wang et al., 1997; Schoch et al., 2002; Schoch et al., 2006). It is a large multi-domain scaffolding protein that was originally discovered as a binding partner of GTP-Rab3A, hence its name (Wang et al., 1997). So far, four genes (Rim1-4) encoding RIMs have been discovered in the mammalian genome. They encode six isoforms (RIM1α, 2α, 2β, 2γ, 3γ, and 4γ) (Wang et al., 2000; Wang and Sudhof, 2003).

RIM1α interacts with Rab3 and Munc13 (Wang et al., 1997; Betz et al., 2001; Wang et al., 2001). Originally it was thought that this binding was mutually exclusive (Betz et al., 2001). However, simultaneous binding can occur (Dulubova et al., 2005). In addition to Rab3 and Munc13, RIM1α interacts with ELKS proteins (also known as Rab6-interacting protein or CAST, also abbreviated as ERC), α-liprins, synaptotagmin and RIM-binding protein (reviewed by Kaeser and Sudhof, 2005). Via the latter, RIM may in turn bind to voltage-gated Ca_v1 and $Ca_v2.2$ Ca^{2+} channels (Hibino et al., 2002), although immunoprecipitation experiments with $Ca_v2.2$ antibodies failed to show co-immunoprecipitation (Khanna et al., 2006; Wong and Stanley, 2010).

RIM1α and Rab3A seem to jointly participate in several forms of presynaptic, PKA dependent LTP (Castillo et al., 2002; Lonart et al., 2003; Huang et al., 2005; Simsek-Duran and Lonart, 2008). RIM1α is likely to be the PKA substrate in the signaling pathway and therefore might function as a phosphoswitch.

Aims and outline of this thesis

This thesis characterizes the function of several neuro-exocytotic proteins in ACh release at the mouse NMJ. For a selection of these proteins it has been investigated whether they play a role in the mechanism underlying homeostatic upregulation of presynaptic ACh release resulting from reduction of the density of functional

postsynaptic AChRs, such as present under myasthenic condition. To this end, NMJ electrophysiology and morphology of several strains of mice genetically deficient for one or more members of neuro-exocytotic protein families has been investigated under normal conditions as well as after inducing experimental myasthenia gravis using α-BTx to block part of the AChRs.

Combined elimination of Munc13-1 and Munc13-2 genes leads to a lethal phenotype around birth. This prevents analysis of Munc13 function in the adult NMJ. In Chapter 2, a study of NMJ function and morphology is therefore performed on Munc13-1/2 double knock-out mouse embryos. In Chapter 3 the effect of genetic deletion of single or multiple isoforms of α-neurexin is reported. Morphological and electrophysiological studies have been performed on soleus muscle NMJ from adult mice and diaphragm NMJs from adult and one day-old mice. This chapter also describes the effect on ACh release homeostasis following application of the toxin model of myasthenia gravis on mice lacking both α-neurexin1 and -2. Chapter 4 reports on the effect of deleting Rab3A on neuromuscular transmission in soleus and diaphragm NMJs. Chapter 5 describes the effect of reducing or increasing Munc18-1 protein levels on exocytotic parameters at the NMJ, in comparison with the effects on cultured glutamatergic and GABAergic central synapses. In view of its modulatory roles, Munc18-1 might be a presynaptic target of the retrograde signals involved in transsynaptic homeostatic upregulation of transmitter release following reduction of postsynaptic AChRs at the NMJ. In chapter 6, the effect of reducing or increasing Munc18-1 protein levels on ACh release homeostasis was investigated in a mouse model for myasthenia gravis. Chapter 7 investigates basic transmitter release properties at NMJs of mice lacking RIM1α as well as the homeostatic response of ACh release to toxin-induced myasthenia gravis. Chapter 8 provides the reader with a general discussion on the findings presented in this thesis and raises some ideas for future research.

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