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Trans-synaptic homeostasis at the myasthenic neuromuscular junction

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TABLE OF CONTENTS

1. ABSTRACT

Properly sustained impulse transmission at the neuromuscular junction (NMJ) is crucial for successful muscle contraction. To guarantee this, NMJs not only possess a considerable safety factor in transmission but also have the ability to adjust the presynaptic acetylcholine release level to cope with any changes in the postsynaptic neurotransmitter sensitivity. This review will provide overview on the discovery and characterization of this synaptic homeostatic mechanism, especially in the condition of the neuromuscular disorder myasthenia gravis (MG) where the postsynaptic transmitter sensitivity at the NMJ becomes severely reduced due to autoimmune attack of acetylcholine receptors. Because homeostatic signalling and adaptation is presumably maximally active in this condition, NMJs from MG animal models are important study objects. Although candidate post- and presynaptic factors as well as the retrograde signals have been proposed, the homeostatic mechanism at the MG NMJ is still incompletely understood. Further identification and functional characterization of key factors is important because these may form new therapeutic targets in MG.

2. INTRODUCTION

The neuromuscular junction (NMJ) is the cholinergic synaptic contact between a motor neuron and a skeletal muscle fibre (1). It has the seemingly simple task to transmit the presynaptic motoneuronal action potential in a one-to-one fashion onto the muscle fibre so that contraction results. Because muscle contraction is so crucial for the organism, the NMJ has evolved to perform its synaptic task failure-free, even during the most intense usage patterns. To this end, NMJs have a large safety factor of synaptic transmission. This means that, due to a high transmitter release level, postsynaptic responses are much larger than minimally needed to exceed the firing threshold of the muscle fibre. Between species and muscle types

large variations exists. For instance, the safety factor at human NMJs is only low (about 2), while in some types of feline NMJs the safety factor can be as large as 12 (2). A safety factor of neuromuscular transmission enables the NMJ to cope with the inevitable rundown of transmitter release which occurs during intense use, when components of the release mechanism become limiting. In addition, a homeostatic mechanism seems present at NMJs with the goal to adjust the presynaptic neurotransmitter release to the actual level of postsynaptic sensitivity for the neurotransmitter, acetylcholine (ACh). In this way reliable transmission remains guaranteed. Most likely, trans-synaptic signals from the postsynaptic membrane are involved. Small changes in postsynaptic neurotransmitter sensitivity can occur during growth, aging or (beginning) disease. However, conditions of the NMJ exist where loss of postsynaptic neurotransmitter sensitivity becomes large and exceeds the boundaries of the physiological range so that (fatigable) muscle weakness results. The most well-characterized disorder is myasthenia gravis (MG) where autoantibodies circulate that are directed to acetylcholine receptors (AChRs) or other postsynaptic factors and thus cause a reduced postsynaptic sensitivity for the transmitter. In this case the compensatory increase of transmitter release induced by homeostatic signalling at the NMJ apparently no longer suffices to counteract the postsynaptic change. Because it is highly likely that in the condition of MG the homeostatic signalling is maximally active, NMJs from MG animal models form interesting study objects to increase knowledge on the molecular factors and mechanisms involved. In the end this may even lead to discovery of therapeutic targets which may be stimulated to attempt to rescue NMJ transmission and thus form a new MG treatment. This review will deal with the discovery and study of homeostatic upregulation of ACh release at the myasthenic NMJ. First, some background overview will be provided on NMJ structure and function and the pathophysiological defects that occur in MG. Synaptic homeostasis is not confined to the mammalian NMJ but rather seems a well-preserved mechanism present at many types of synapses amongst many different species. Especially well studied in this respect is the larval NMJ of the fruit fly, *Drosophila*. In this review, only those aspects of the *Drosophila* studies with the most relevance to the mammalian NMJ will be discussed. For complete overview on *Drosophila* NMJ homeostasis, the reader is referred to some excellent recent reviews (3, 4).

3. MORPHOLOGY AND FUNCTION OF THE NEUROMUSCULAR JUNCTION

3.1. Morphology

Skeletal muscles are innervated by a peripheral nerve containing the axons which belong to motor neurons residing in the spinal cord. In the muscle, mostly in a well-defined midline region (Figure 1A), these myelinated axons display some degree of branching and each terminal is forming a synaptic contact with just one muscle fibre. A particular motor neuron, its axon, the NMJs it forms and all the connected muscle fibres form a functional unit termed the 'motor unit'. Muscles needed for delicate movements generally have small motor units, i.e. the motor neuron innervates only a small number (tens) of muscle fibres. At the terminal, a motor axonal branch loses its myelin. Instead, a few perisynaptic Schwann cells cover the synaptic structure. These cells provide structural and trophic support and may even modulate synaptic transmission, although not all aspects are well understood yet (5). In addition a fourth cell type has been described, i.e. a fibroblast-like NMJ capping cell called kranocyte (6). However, its role has remained enigmatic so far. Interestingly, very recently it has been shown that most NMJs are closely contacted by axon terminals of sympathetic neurons and that these seem to play a role in synaptic maintenance (7). The motor axon terminal forms a presynaptic branched structure that neatly follows the contours of the postsynaptic AChR clusters in the postsynaptic membrane. The pre- and postsynaptic membranes are separated by a ~50 nm wide synaptic cleft. Considerable inter-species variation of axon terminal morphology and thus NMJ morphology exists, with human NMJs being much smaller than those of rodents (8). In most mouse and rat muscles the nerve terminal, and hence the opposed AChR area, forms a structure which appears as a 'pretzel' (Figure 1B). However, human axon terminals, as well as those of many other species in the animal kingdom, consist of multiple small boutons which are connected by miniscule branches (9). The synaptic interface of the motor axon terminal harbours 'active zones' at the cytoplasmatic side of the membrane where a molecular machinery is concentrated that governs neuroexocytosis, together with a docked pool of clear synaptic vesicles containing the neurotransmitter ACh. Generally, ~2.5 active zones are present per μm^2 presynaptic membrane and this density is maintained during most of the lifetime of an NMJ (10). Each motor nerve terminal contains ~150,000-300,000 synaptic vesicles which each are filled with a 'quantum' of ACh (~10,000 molecules). Voltage-gated Ca^{2+} channels ($Ca_v2.1$ type in mammals) are localized in the presynaptic membrane at the active zone. Their opening in response to the presynaptic action potential invading the motor nerve terminal causes influx of Ca^{2+} ions, driven by the large concentration difference outside and inside the presynaptic nerve terminal (~2 mM and ~200 nM, respectively). Knowledge on the molecular constituents that form the neuroexocytotic machinery and there structural interactions at active zones has increased spectacularly in recent decades and has been reviewed by others (e.g. (11, 12)). Once released, ACh diffuses over the ~50 nm synaptic cleft and binds postsynaptic AChRs which translate the

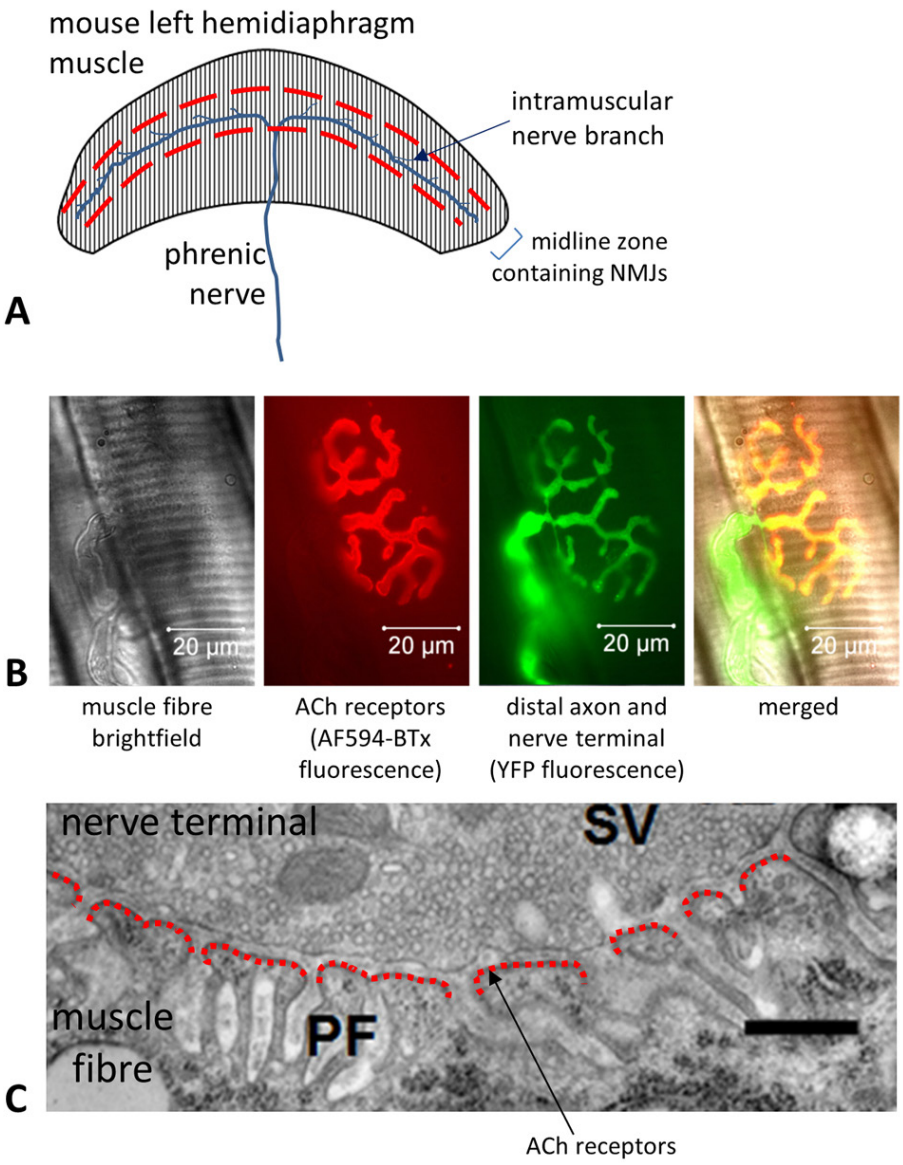

Figure 1. Morphology of the NMJ. A) Schematic drawing of a left hemidiaphragm from the mouse, a muscle-nerve preparation often used for functional NMJ studies. View from the thoracic side, with indication of the midline zone that harbours the NMJs and the innervating phrenic nerve. B) Light- and epifluorescence microscopical pictures of a single levator auris longus muscle NMJ from a mouse expressing YFP (green) in motor neurons, and with ACh receptors stained red with AlexaFluor594-conjugated α-bungarotoxin (AF594-BTx). A high degree of co-localisation is observed of these pre- and postsynaptic stainings. C) Electron microscopical picture of a part of an NMJ, with the many ACh containing synaptic vesicles (SV) localised in the nerve terminal. Postsynaptic folds (PF) are visible in the muscle membrane. ACh receptor localization is indicated with red dots. Scale bar is 0.5 µm.

chemical into an electrical signal. The ACh is removed by hydrolyzation by acetylcholinesterase within the synaptic cleft. The synaptic cleft is lined with basal lamina which contains synapse-specific extracellular matrix proteins such as agrin, particular laminins, perlecan, collagen-IV, -XIII and -Q (13).

The muscle fibre membrane at the NMJ is extensively folded (Figure 1C), and the nicotinic AChRs reside at high density of ~10,000/µm^2 on their tops, opposed the presynaptic active zones. AChRs are heteropentameric ligand-gated ion channels consisting (in adult NMJs) of two α, one β, one δ and one ε subunit. ACh binds to the α subunit, at the interface with a non-α subunit. When both α subunits of an AChR are bound, the channel pore opens (14). The ion channel is relatively non-specific, it conducts the cations Na^+ and K^+, and some Ca^{2+}. Interestingly, human AChRs allow for a higher Ca^{2+} permeability than mouse AChRs (~7 and 4% of the total current, respectively) (15). In the bottoms of the folds, $Na_v1.4$. type voltage-gated Na^+ channels are present at ~10 fold higher density than elsewhere in the sarcolemma. This increases the membrane excitability at the NMJ

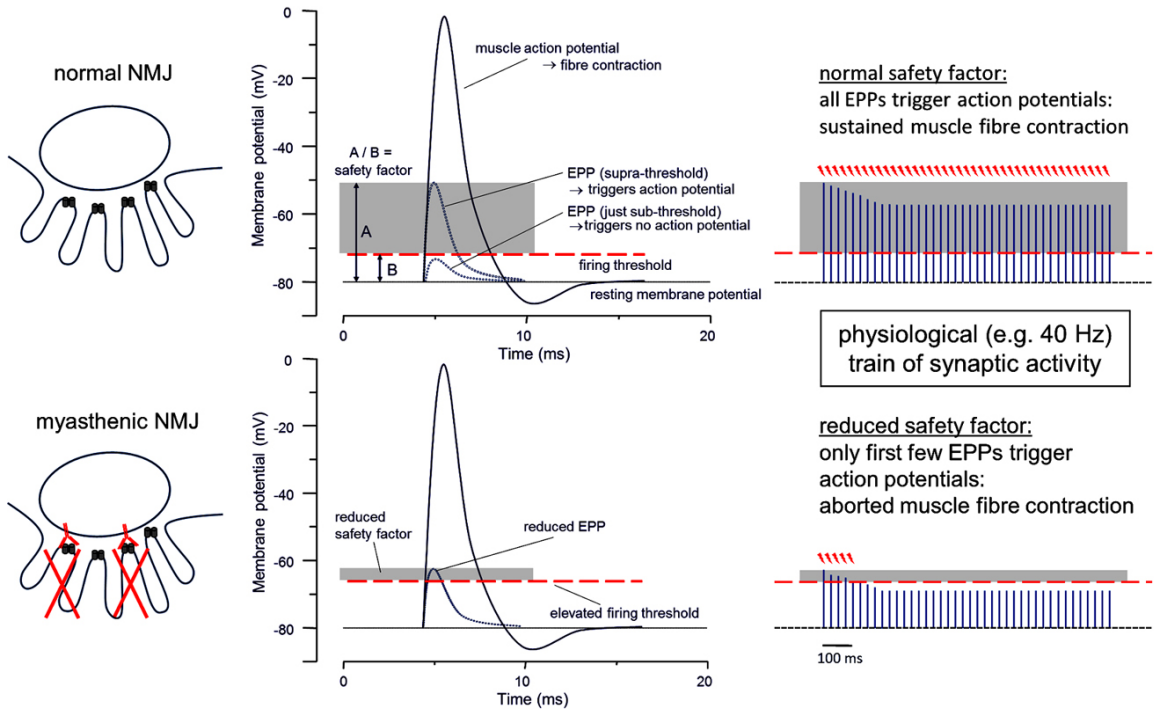

Figure 2. Schematic explanation of the electrophysiological function of a healthy NMJ (top row) and a myasthenic NMJ (bottom row). Normal NMJs have a high safety factor of neuromuscular transmission, guaranteeing that triggering of muscle fibre action potentials transmission (indicated as red flashes in the right-hand panels) is sustained during high rate synaptic activity and causes tetanic contraction. In this way the NMJ can easily cope with the rundown of endplate potential (EPP) amplitude during intense activity. In the myasthenic NMJ, autoantibodies reduce ACh receptors and cause complement-mediated damage to the postsynaptic membrane. This leads to smaller EPPs and elevated firing threshold, together resulting in a reduced safety factor of neuromuscular transmission. This results in transmission failure upon intense use of the synapse, explaining the fatigable muscle weakness.

and therewith lowers action potential firing threshold (8, 16). The narrow cytoplasmatic space forms a high resistance pathway for the ACh-induced membrane ion current. This amplifies the depolarizing response and facilitates in this way the triggering of a muscle fibre action potential (17).

3.2. Function

When the motor axonal action potential progresses into the nerve terminal it stimulates opening of $Ca_v2.1$ channels. The resulting Ca^{2+} influx triggers simultaneous exocytosis of a number of ACh quanta from several active zones. The released amount of quanta (the 'quantal content'), is dependent on nerve terminal size and hence on the number of active zones. This parameter varies considerably amongst NMJs of different muscles and species. NMJs of humans are normally quite small (~100 μm^2) and have a quantal content of ~20 (18). Mouse and rat NMJs are on average >2-fold larger and have a higher quantal content, of ~40-100 (1, 9). The released amount of ACh opens many thousands of AChR channels and causes a dominant Na^+ influx, accompanied by some Ca^{2+} influx and K^+ efflux. The net result is a inward ionic endplate current (EPC) which causes a local depolarization of the membrane, forming the endplate potential (EPP). This EPP has an amplitude of ~15-30 mV, depending on muscle type and species. The resting membrane potential of skeletal muscle fibres is around -80 mV. The EPP stimulates opening of synaptic $Na_v1.4$. type voltage-gated Na^+ channels. The density and characteristics of these channels dictate the firing threshold, i.e. the depolarization level at which exactly that many Na^+ channels open so that a regenerative process of channel opening and further depolarization ensues, forming the rising phase of the muscle fibre action potential. The action potential travels away in two directions from the NMJ over the muscle fibre membrane. They invade the T-tubular system of the muscle fibre and trigger the excitation-contraction system which elicits contraction of the muscle fibre (19).

EPPs in rat and mouse NMJs are in the range of ~20-35 mV, which is much larger than the ~10-12 mV minimally needed to trigger an action potential (20, 21). Hence, a safety factor of neuromuscular transmission is present in healthy muscle (Figure 2), which varies considerably amongst mammal species. In mouse and rat NMJs it varies from 1.8 to 6, while at human NMJs the safety factor is only ~2 (2). A safety factor at the NMJ ensures successful transmission even during times of intense use, when the quantal

content tends to decrease due to presynaptic factors becoming limiting (e.g. $Ca_v2.1$ channel inactivation and vesicle replenishment). Sustained tetanic muscle contraction results when a motor neuron fires trains of nerve action potentials in the range of 20-100 Hz, which depends on muscle fibre types (22, 23). During tetanic activity, EPP amplitudes at mouse NMJs diminish by 20-30% to a more or less constant level after the first 10 impulses (24). At human NMJs there is even a deeper run-down of EPPs, by ~40% (25). In spite of this, EPP amplitudes remain suprathreshold due to the existing safety factor of transmission (Figure 2). However, diseased conditions exist when the safety factor becomes compromized, see below.

Besides nerve stimulation-evoked multi-quantal ACh release, nerve terminals now and then spontaneously release a single ACh quantum. Each quantum opens a limited number of AChRs which causes a miniature EPP (MEPP) at the muscle fibre membrane. Their frequency varies with muscle type and is correlated with the total NMJ size. At mouse NMJs, the MEPP frequency is ~1-4/s, at the small NMJs of human intercostal muscle it is much lower, i.e. <0.1/s (18, 25). Mean MEPP amplitudes at NMJs range from about 0.3 to 1.5 mV. This depends on multiple factors which vary per muscle type, e.g. the electrical input resistance of the muscle fibre, which is inversely correlated with diameter. MEPP rise and decay kinetics are roughly similar to those of the EPPs. MEPP amplitudes are normally sub-threshold and thus do not trigger a muscle action potential. Their physiological function at the mammalian NMJ is still rather unclear. Perhaps MEPPs are just random spill-overs from the huge number of synaptic vesicles (several hundred-thousands) present in the motor nerve terminal (26). However, in other types of synapses it has been suggested that spontaneous uni-quantal synaptic events can influence postsynaptic local protein synthesis (27), and may be necessary for structural synapse maturation (28). It remains to be seen whether MEPPs have such roles at mammalian NMJs.

4. MYASTHENIA GRAVIS AND ITS ANIMAL MODELS

4.1. Myasthenia gravis

MG is an autoimmune disorder hallmarked by fluctuating and fatigable muscle weakness. For clinical and therapeutic overview see (29). The muscle weakness is caused by a defect of the NMJ resulting from autoantibody attack. The exact triggers and susceptibility factors for developing autoimmune MG are largely unknown (30). In most (~85%) of the MG patients, autoantibodies are present which are directed against the AChR. These are generally of the IgG1 and IgG3 isotype. An important effect is activation of the complement cascade which in the end locally destroys the postsynaptic membrane, thereby removing AChRs but also synaptic $Na_v1.4$. channels. In addition, the autoantibodies can cross-link AChRs, which speeds up their endocytosis and degradation. Furthermore, direct ion channel-blocking actions on AChRs have been postulated. The electrophysiological result of these effects is that EPPs become severely reduced and that the muscle fibre firing threshold is elevated (1). In this way the safety factor of neuromuscular transmission becomes severely compromized (Figure 2). When there is intense use of the NMJ this can lead to progressive block of neuromuscular transmission which then results in the characteristic myasthenic fatigable muscle weakness. In smaller proportions of the MG patients, autoantibodies against the postsynaptic proteins muscle-specific kinase (MuSK) or low-density lipoprotein receptor-related protein 4 (LRP4) have been demonstrated. The role of LRP4 is to bind neurally released agrin and then to activate MuSK, which in turn causes AChR clustering (31). Anti-agrin antibodies have also been detected in a few MG patients, often with co-seropositivity for AChR, MuSK or LRP4 antibodies (32, 33). MuSK MG has distinct clinical features and the autoantibodies are generally of the IgG4 class (34). The antibodies of this special isotype are hetero-bispecific and do not activate complement. Passive transfer into mice causes muscle weakness due to severe fragmentation of the AChR area of NMJs (35, 36). The molecular mechanism is largely unknown but likely involves block of MuSK-LRP4 interaction by the anti-MuSK antibodies (37, 38). The pathophysiological actions of MG autoantibodies against LRP4 and agrin are still elusive but most likely eventually lead to removal of postsynaptic AChRs, too.

4.2. Animal models of myasthenia gravis

For detailed overview on MG animal models and the methods and techniques to study their NMJ function see (1, 39, 40). The first animal model for AChR MG was developed more than 40 years ago by injecting rabbits with purified AChRs from electric eel (41). The animals developed muscle weakness and antibodies against AChRs appeared in the serum. Since then, many different species-variants of this active immunization model have been developed and applied to study disease mechanisms and therapeutic action of new compounds, including complement inhibitors (42). Another method of generating an MG animal model is to inject MG patient serum, purified patient antibodies or monoclonal antibodies against the AChR. With this so-called passive transfer method, myasthenic symptoms have been induced in e.g. mice, rats and guinea pigs. In addition, a non-immunological rat or mouse model with (mild) myasthenic symptoms of muscle weakness can be generated by injecting repetitive low doses of the purified snake toxin α-bungarotoxin (α-BTx), a near-irreversibly AChR

antagonist, to obtain block of a proportion of the AChRs (43, 44). With small adjustments of the daily dosing, according to the severity of the symptoms, a well-controlled stable disease level could be achieved which was termed toxin-induced MG (TIMG).

5. HOMEOSTATIC PLASTICITY AT THE MYASTHENIC NEUROMUSCULAR JUNCTION

5.1. Historical background

First indications that MG NMJs respond with presynaptic compensatory changes to the postsynaptic reduction of their AChR density came from biochemical studies in the 1970s. The nerve endings in intercostal muscle biopsies from MG patients appeared to contain an about two-fold higher amount of ACh as muscles from non-MG controls (45). In further biochemical studies on MG patient muscle biopsies and experimental autoimmune MG rat muscles, increases were shown in the ACh content and the initial phase of ACh release induced by high K^+ depolarization (46). In agreement with these findings, the level of the ACh synthesizing enzyme choline acetyltransferase was shown increased in muscles from MG patients, experimental MG rats and rabbits (47, 48). However, this was not confirmed in a later study on experimental autoimmune MG rat diaphragm muscles (18). Morphologically, an increased density of presynaptic ACh vesicles was demonstrated in electron microscopical pictures of motor nerve terminals of experimental MG rat forelimb muscles (1, 49). At human MG NMJs, however, such an effect was not observed (50). Micro-electrode recordings allow for very detailed study of NMJ function at individual synapse level, including neurotransmitter release levels (1). With this method the biochemical indications that ACh release is increased at myasthenic NMJs were further substantiated. Quantal contents of NMJs from MG intercostal muscle biopsies, as determined from EPP and MEPP amplitudes, were increased by a factor 3-4 (51). The dependency of the quantal content on the extracellular Ca^{2+} concentration was explored. It appeared that at Ca^{2+} concentrations lower than 0.7 mM the ACh release was increased ~5 times at MG NMJs and that this difference became smaller at higher Ca^{2+} concentrations. It should be noted that these electrophysiological measurements were done in the presence of an acetylcholinesterase inhibitor to increase the small MEPPs so that they were easier to detect. The high ACh concentrations in the synaptic cleft may potentially have influenced the presynaptic release, e.g. by stimulating putative muscarinergic presynaptic ACh autoreceptors (52). Others used the 'cut-fibre' method in experimental animal muscle, i.e. cutting the muscle fibre ends to cause some depolarization so that the fibres do no longer produce action potentials and contraction. In this way quantal contents were measured (without using acetylcholinesterase inhibition) at NMJs of actively immunized MG rats and found to be increased by ~20% (53). However, because the MEPP amplitude depends on the resting membrane potential, it is highly likely that they missed the smallest MEPPs and thus underestimated quantal contents. In our own laboratory we have applied μ-conotoxin GIIIB in the electrophysiological investigation of myasthenic mouse and rat NMJs since the early 1990's (54). This toxin of the tropical marine snail *Conus Geographus* selectively blocks the $Na_v1.4$. channels on skeletal muscle fibres and thus prevents the muscle fibre action potential and the resulting contraction. It thus enables undisturbed recording of EPPs at normal resting membrane potential (55). In this way the very small MEPPs at myasthenic NMJs can be recorded and quantal contents can be calculated in the 'direct' way (i.e. division of mean EPP amplitude by the mean MEPP amplitude) at single NMJs (18). Using this method we have analyzed ACh release at NMJs of TIMG rats in great detail. First, we showed that the α-BTx treatment of TIMG rats reduced the average MEPP amplitude in diaphragm NMJs by ~40% and caused an increase of the average quantal content by ~50%. Importantly, at each single NMJ the extent of increase in quantal content was inversely related to MEPP amplitude (Figure 3). This indicated the existence of a local homeostatic synaptic mechanism using retrograde signals, because otherwise, in case of a general effect, even NMJs with MEPP amplitudes in the range of normal would have had upregulated quantal content (54). Further study of the TIMG model revealed that the increase in quantal content is not instantaneous; it develops gradually to a more or less stable plateau level reached after 3-4 weeks α-BTx treatment. Furthermore, the increase in quantal content clearly depends on the extracellular Ca^{2+} concentration and is no longer present at concentrations lower than 1 mM (56). Because low extracellular Ca^{2+} has been used as a method to reduce ACh release to prevent muscle contraction to be able to record MEPPs and EPPs, this may have obscured observation of clear quantal upregulation in earlier studies in actively immunized MG as well as TIMG rats (43, 57). *Ex vivo* pharmacological investigations using specific inhibitors of candidate ion channels and signalling cascade factors revealed that the compensatory increase in quantal content at TIMG rat NMJs did not involve presynaptic Ca_v1 or $Ca_v2.2$. Ca^{2+} channels or K^+ channels, but that Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) and Trk tyrosine kinases (which form receptors for neurotrophic factors) may be involved (56, 58). The upregulation of ACh release at (TI)MG NMJs could in principle be due to an increase in motor nerve terminal size and a concomitant increase in the number of active zones. It is known that motor nerve terminal size positively correlates with quantal content and the amount of active zones (8, 59, 60). However, morphological analysis of TIMG

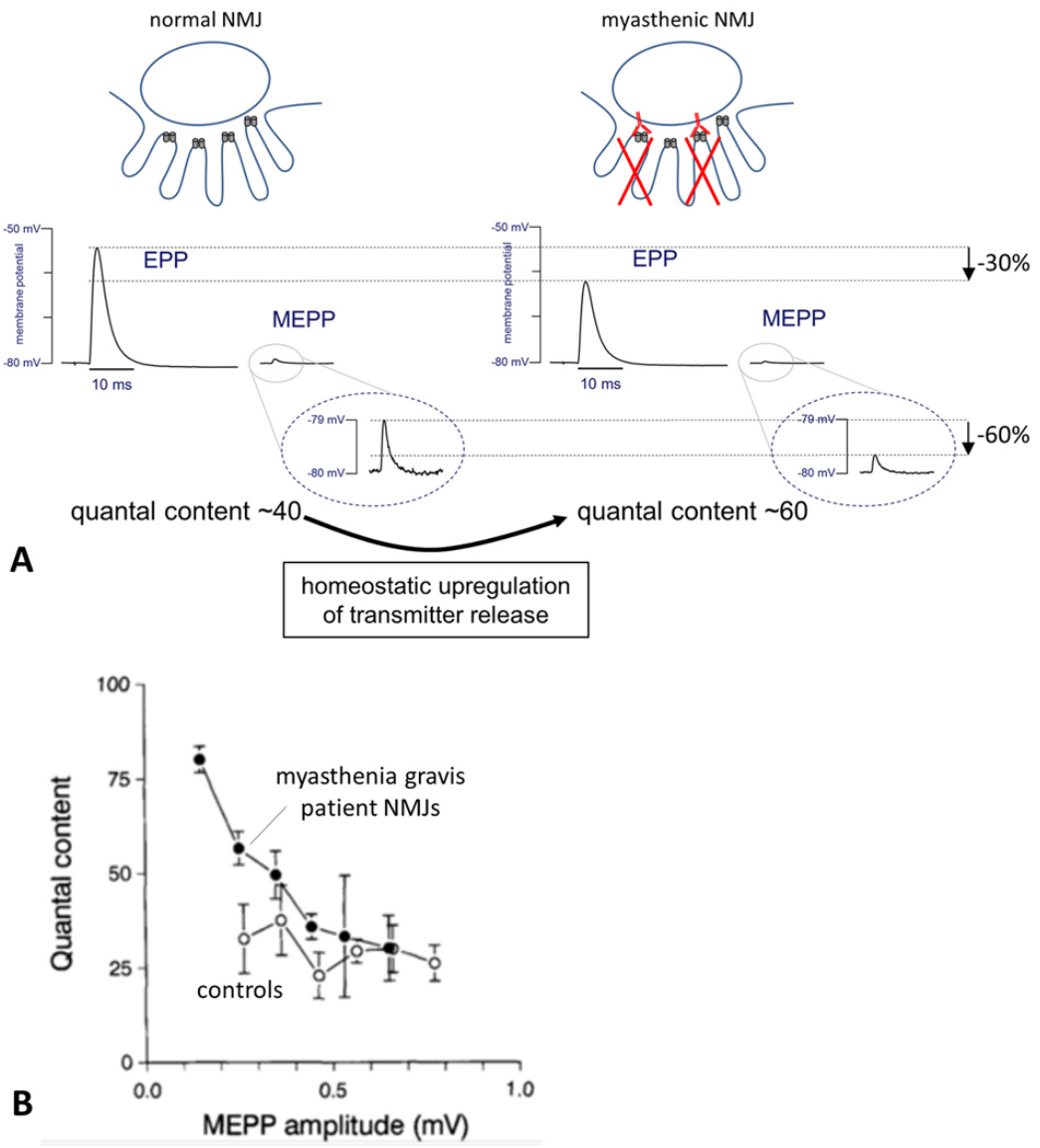

Figure 3. Schematic illustration of the electrophysiology of the myasthenic NMJ, with homeostatic upregulation of presynaptic ACh release. A) Reduction of ACh receptors by the autoantibodies leads to reduction of the uniquantal miniature endplate potential (MEPP) amplitude, in this example by ~60%. The multiquantal EPP amplitude, however, becomes reduced by only ~30%. This shows that there is upregulation of the quantal content, the amount of ACh quanta released per nerve impulse, in this example by ~50%. B) An inverse relationship between quantal content and MEPP amplitude at the level of individual human myasthenic NMJ from intercostal muscle biopsies illustrates homeostatic adaptation of quantal content in myasthenia gravis. Reproduced with permission from (18). A similar inverse relationship has been demonstrated in several MG animal models, see text.

rat NMJs excluded such an increase in NMJ size; the area delineated by staining for acetylcholinesterase and the nerve terminal marker synaptophysin was found unchanged (43)(J.J. Plomp unpublished data). The phenomenon of upregulated quantal content at the level of single NMJs appeared not limited to TIMG; it was confirmed in intercostal muscle biopsy NMJs from AChR MG patients as well as in diaphragm NMJs from actively immunized AChR MG rats (18). On average, the quantal content of human intercostal NMJs was roughly doubled as compared to controls (from ~30 to ~60), but there was quite some variation between MG patients (the mean value varied from 36 to 84). As in TIMG, a clear inverse correlation was confirmed between the mean MEPP amplitude and the quantal content of single NMJs (Figure 3B). In actively immunized AChR MG rats, the mean quantal content was increased ~3-fold and a similar inverse relationship between quantal content and MEPP amplitude at individual NMJ level was demonstrated (18). These results have been further confirmed in recent passive and actively immunized AChR MG mice (61). Quantal contents are generally determined from EPPs recorded at low nerve stimulation rate (i.e.

<1 Hz), at which there is no or almost no amplitude rundown. However, upon high rate nerve stimulation in the physiological usage range (e.g. 40 Hz), quantal content and thus EPP amplitude runs down by ~20-30% to a plateau level reached after the first ten impulses. At myasthenic rat NMJs with established upregulation of quantal content the extent of this EPP amplitude rundown was consistently found to be more pronounced, i.e. the EPP amplitude was depressed by ~40-50% (18, 56). At human MG NMJs, EPPs also run down to a lower level than at control NMJs, i.e. by ~60 and ~40%, respectively (25).

The compensatory homeostatic mechanism of quantal content upregulation at the NMJ does not seem to require *direct* AChR removal or block by external factors such as antibodies or α-BTx. NMJs of heterozygous neuregulin *null*-mutant mice have reduced postsynaptic AChR density and also display compensatory increased presynaptic ACh release (62). Similarly, the phenomenon of compensatory increase in quantal content has been shown in a few human congenital MG cases with mutations in the gene coding for the ε-subunit of the AChR, leading to reduced AChR density and, consequently, small MEPPs (63).

5.2. Further phenotypical details of the homeostatic quantal content upregulation at the neuromuscular junction

In more recent years, additional aspects on the homeostatic upregulation of transmitter release at myasthenic NMJs have been revealed. Local induction of TIMG in mice by injection of α-BTx at the tibialis anterior muscle caused reduction of functional AChRs when assessed after five days (64). The quantal content was found more than doubled and statistical analysis revealed that the number of releasable vesicles (*n*) had gone up, rather than the probability of release (*p*). The quantal content is assumed to be the product of *n* and *p* (9, 65, 66). Interestingly, the quantal content increasing effect of α-BTx treatment could also be brought about at NMJs of muscles whose innervating nerve was blocked by tetrodotoxin during the induction period (64). This shows that presynaptic nerve spiking activity and, consequently, evoked ACh release is not required to induce the quantal content increase. Rather, AChR activation by spontaneous quantal ACh release or background non-quantal ACh release, which are not blocked by tetrodotoxin, may be used by the postsynaptic component of the homeostatic machinery to sense the neurotransmitter sensitivity. The increase in *n* was not due to enlargement of motor nerve terminals, although in a small proportion (~7%) of the investigated NMJs some degree of terminal sprouting was observed. However, no matching AChR staining was present at these sprouts so it is not likely that they contributed to the observed increase of quantal content. The sensitivity of TIMG-upregulated ACh release to the $Ca_v2.1$ channel blocker ω-agatoxin-IVA was not different from normal control NMJs, suggesting that there is no upregulation of other types of Ca_v channels which causes the increased ACh release. In a very recent study of the same group (67), it was shown that *in vitro* application of a short (2 min) pulse exposure of mouse tibialis anterior muscle to a very high concentration (1 mg/ml) of α-BTx induced reduction of upregulation of quantal content came about almost instantaneously, i.e. it was present when the electrophysiology of NMJs was assessed 1 h after α-BTx exposure. This result differs from earlier observations that 3 h after *in vivo* intraperitoneal injection of α-BTx in rats no increase in quantal content was present at diaphragm NMJs in spite of a considerable reduction in the postsynaptic sensitivity to ACh (54). As yet, the reasons for this difference remain unclear. On the basis of further pharmacological studies and statistical analyses, Wang *et al*. (67) conclude that homeostatic upregulation of quantal content is due to Ca^{2+}-dependent recruitment of a small reserve pool of vesicles that normally have slow-release kinetics. Following activation of this homeostatic reserve pool, it is released synchronously with the normal readily releasable pool of ACh vesicles. The small reserve pool of vesicles is depleted quite rapidly which might explain the extra EPP depression during high rate nerve stimulation at NMJs with upregulated quantal content (18, 56, 64).

As mentioned above, a variant of MG exists in which autoantibodies against MuSK are present. Passive transfer studies have demonstrated that these (IgG4) MuSK antibodies are directly pathogenic and eventually cause disruption of the postsynaptic AChR area at the NMJ which results in severe reduction of the AChR density, causing failure of synaptic transmission (1, 35, 36). Most interestingly, no compensatory upregulation of quantal content was found at compromized NMJs of MuSK MG IgG4 passive transfer mice as well as NMJs from a MuSK MG patient in an intercostal muscle biopsy (25, 36). Absence of compensatory increased quantal content and complete loss of the inverse relationship between MEPP amplitude and quantal content of single NMJs was shown in further MuSK MG passive and active immunization mouse studies (61, 68-70). This implies that MuSK, or an interacting factor, may be involved in the homeostasis pathways that sense AChR loss or release retrograde messaging molecules. Interestingly, some MuSK-signalling pathway members, such as agrin and rapsyn, interact with or take part in a postsynaptic glycoprotein complex clustered around dystrophin, and deletions from this complex can affect NMJ structure, function and synaptic homeostasis (71). Alternatively, absence of an appropriate homeostatic response at MuSK MG NMJs may be due to secondary presynaptic damage

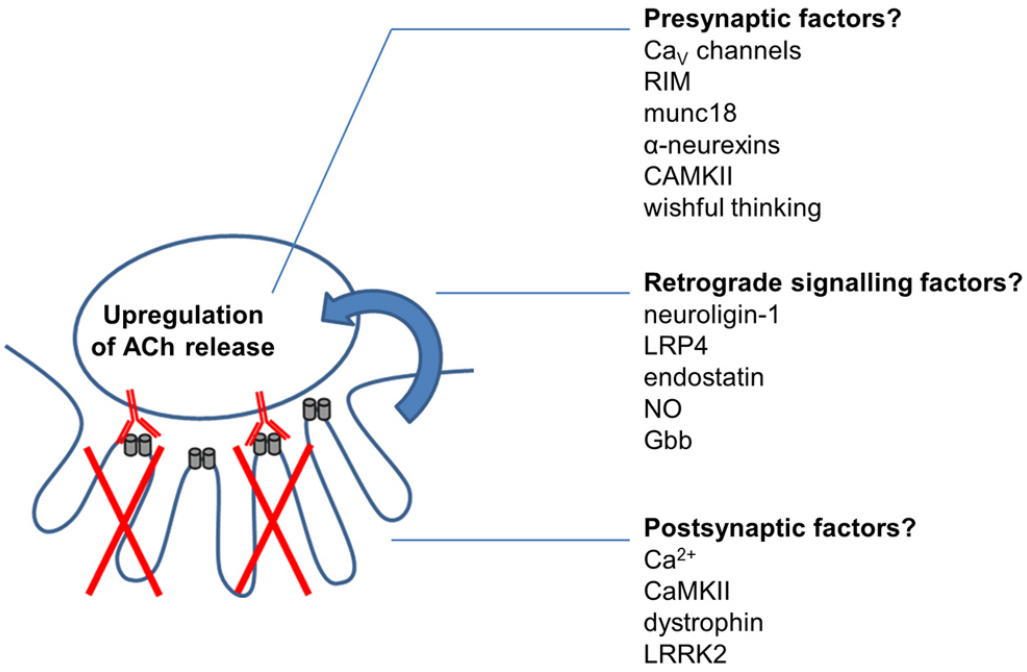

Figure 4. Summary illustration of the pre- and postsynaptic factors and retrograde signalling factors that may be involved in homeostatic upregulation of ACh release at the myasthenic NMJ. For detailed information and references on each of these factors, see text.

resulting from the severe postsynaptic disruption, preventing the nerve terminal to respond to retrograde signals. Furthermore, misalignment of pre- and postsynaptic membrane specializations (35) may be relevant here because if the total nerve terminal would in fact release extra ACh, a partial lack of opposing postsynaptic AChR area would obscure this. In any case, lack of upregulated functional ACh release at MuSK MG NMJs probably renders transmission more vulnerable to AChR loss, as compared to AChR MG NMJs with adequate upregulation.

5.3. Studies into the molecular mechanism of the homeostatic quantal content upregulation at the neuromuscular junction

While the phenotype of the homeostatic response of mammalian NMJs to the condition of (experimental) MG has been described quite well, the identities of the postsynaptic sensors, the retrograde messenger(s) and the presynaptic targets involved have so far remained mostly elusive. Below, the most important candidate factors will be discussed (Figure 4).

5.3.1. Presynaptic targets

Transmitter release at mammalian motor nerve terminals is predominantly governed by Ca^{2+} influx via $Ca_V2.1$ channels (72). These channels are thus likely targets for a putative retrograde factor to stimulate and thus induce increased quantal content. In *Drosophila* larval NMJ studies it was suggested that presynaptic $Ca_V2.1$ channels indeed may be involved. The *Drosophila* variety of the pore-forming subunit of this channel is encoded by the *cacophony* gene. A point mutation in this gene renders the *Drosophila* larval NMJ unable to develop a homeostatic increase in quantal content in response to philanthotoxin (PhTx)-induced partial block of postsynaptic receptors (which in *Drosophila* NMJs are in fact glutamate receptors)(73). Further analyses showed that increased presynaptic Ca^{2+} influx underlies the homeostatic increase of quantal content at the Drosophila NMJ (74). However, as yet there is no convincing evidence for $Ca_V2.1$ nor other types of Ca_V channel involvement in quantal content upregulation at mammalian NMJs. Acute *ex vivo* application of blockers of Ca_V1 and $Ca_V2.2$. type channels did not reduce upregulated quantal content at rat TIMG NMJs (35, 56). Furthermore, NMJs with acutely upregulated quantal content induced by *in vitro* α-BTx application show normal sensitivity to the specific $Ca_V2.1$ blocker ω-agatoxin-IVA (64). Another relevant presynaptic factor suggested from *Drosophila* NMJ studies is Rab3A-interacting molecule (RIM) (75). RIMs in mammalians are encoded by four genes which produce scaffolding proteins that help to insert and keep several protein constituents of the active zone (e.g. $Ca_V2.1$ channels) in the correct position for optimal function in the process of neurotransmitter release (76). *Drosophila* possesses only one RIM gene and its knock-down, knock-out or specific point mutation renders the larval NMJ unable to respond with a homeostatic increase of quantal content to block of part of the postsynaptic glutamate receptors

by PhTx (74). In addition, it was shown that RIM acts by increasing the readily releasable pool of transmitter vesicles, rather than by stimulating presynaptic Ca^{2+} influx. In our own laboratory we tested whether the mammalian isoform RIM1α is involved in homeostatic quantal content upregulation at the mouse NMJ. Central synapses of RIM1α knockout mice show altered synaptic plasticity (76). However, NMJs of RIM1α knockout mice showed normal compensatory upregulation of quantal content after TIMG treatment (M.S. Sons and J.J. Plomp, unpublished data). One reason may be that absence of RIM1α is compensated for by the highly homologous isoform RIM2α.

We also studied a possible role of the neuroexocytotic protein Munc18, which interacts with members of the SNARE protein complex responsible for regulated neuroexocytosis and is crucial for neurotransmitter release (77). Munc18 level has been shown to be a determinant of the size of the readily releasable pool of synaptic vesicles in nerve terminals (78). We applied TIMG treatment to heterozygous Munc18 null-mutant mice, which have 50% Munc18 protein level, and observed that their NMJs were less well capable to upregulate quantal content (44). This result is in agreement with the observations that Munc18 levels influence the size of the readily releasable pool of synaptic vesicles (78), and that an increase in this size underlies homeostatic upregulation of quantal content at NMJs when a proportion of the AChRs is blocked (67).

α-Neurexins are further interesting candidate presynaptic proteins to mediate upregulation of quantal content at myasthenic NMJs. In mammals, three genes exist that encode α-neurexin isoforms; alternative splicing leads to many variants. They function as presynaptic cell adhesion molecules which interact with many other synaptic proteins, such as neurexophilin, neuroligin and synaptotagmin (79). We induced TIMG at wild-type and double-knockout mice (which lacked either α-neurexin-1 and -2 or α-neurexin-2 and -3) and showed that the upregulation of quantal content at their diaphragm NMJs was considerably less outspoken than when TIMG was induced in normal controls (80). Possibly, α-neurexins mediate homeostatic upregulation of quantal content via an (indirect) influence on $Ca_V2.1$ channels. The exact mechanism remains unclear but may involve interactions with other active zone proteins like CASK and Mint (79).

5.3.2. Candidate retrograde signalling molecules

As yet, no retrograde signals have been firmly identified, but several interesting candidates have been suggested from *Drosophila* NMJ studies and experiments on synapses of cultured mouse neurons. An interesting candidate is postsynaptic neuroligin-1, a single-pass transmembrane protein which in central synapses has been shown to form trans-synaptic complexes with presynaptic neurexins (81). In cultured mouse brain cortex glutamatergic synapses it appeared that synaptic activity stimulates the local proteolytic activity of matrix metalloprotease 9 (MMP9) which causes cleavage of postsynaptic neuroligin-1 (82). This destabilizes presynaptic neurexin which results in a reduction of the presynaptic transmitter release. This MMP9-mediated cleavage of neuroligin-1 could be abrogated by co-application of the NMDA glutamate receptor blocker AP5. A similar tuning mechanism comprising of neuroligin, neurexin and MMP9 might be hypothesized to underlie retrograde signalling in the upregulation of quantal content at the myasthenic NMJ. Interestingly, it has been shown *in vitro* that LRP4, an important postsynaptic molecule at the mammalian NMJ, can also be cleaved by MMP9 and that the extracellular domain is shed into the extracellular space (83, 84). Moreover, the extracellular domain of LRP4 appears capable of binding to developing motoneuronal membrane where it induces clustering of active zone molecules and synaptic vesicles, suggesting that LRP4 may act as a retrograde messenger at NMJs (85). If this system would remain active in the adult NMJ, it might play a role in the compensatory upregulation of quantal content at myasthenic NMJs. Of note, the inability of MuSK MG NMJs to upregulate their quantal content may be thus be explained by LRP4 being such an intimate partner of MuSK and therefore perhaps vulnerable to collateral damage when autoantibodies attack MuSK. Intriguingly, LRP4 has also been proposed to be present presynaptically, also with modulating roles. It is clear that the role(s) of LRP4 in NMJ formation, function and homeostasis are very interesting and potentially of importance, but have to be further elucidated in extended studies.

The peptide endostatin is another candidate retrograde signal suggested from *Drosophila* NMJ studies (86). This fragment is proteolytically cleaved from multiplexin, the *Drosophila* homologue of mammalian collagen XVIII, a ubiquitous extracellular matrix molecule. Mammalian endostatin is mainly known for its potent anti-angiogenic properties and can be formed by cleavage of collagen XVIII by MMP9 (87). It appeared that homeostatic upregulation of quantal content at *Drosophila* larval NMJs in response to partial glutamate receptor block by mutation or PhTx treatment was absent in mutants which lacked the endostatin terminal fragment in their multiplexin or mutants that lacked the entire multiplexin (86). Endostatin has been hypothesized to act on presynaptic $Ca_V2.1$ channels to increase Ca^{2+} influx. As yet, it has not been investigated whether endostatin plays a role in homeostasis the mammalian NMJ.

From the study of synaptic plasticity in hippocampal and cortical synapses, nitric oxide (NO) has been suggested as a retrograde signalling molecule (88). Very low levels of NO synthase-1 (NOS1) are present at postsynaptic membranes. This produces very small amounts of NO which are locally released into the synaptic cleft. NO then increases presynaptic transmitter release through a stimulatory action on presynaptic ion channels and neuroexocytotic SNARE proteins. In hippocampal slice synapses, application of NO scavengers such as hemoglobin reduce or block synaptic plasticity. The relevance for the NMJ remains to be seen. No detailed studies have been performed; in a preliminary study in our laboratory, hemoglobin application to rat TIMG NMJs did however not reduce the upregulated quantal content (J.J. Plomp, unpublished data).

Final candidates for retrograde signalling factors suggested from *Drosophila* experiments are secreted bone morphogenetic protein (BMP) pathway members (89). One of them, *glass bottom boat* (Gbb), is a muscle-derived retrograde growth factor for the presynaptic nerve terminal (90). Although Gbb seems needed to make the presynaptic nerve terminal competent for induction of homeostatic upregulation of quantal content, it appears not to be the actual instructive messenger (91). Other BMP pathway signalling factors might play that role, presumably via the binding to the presynaptic BMP receptor *wishful thinking*. Whether or not BMP signalling plays a role in mammalian NMJ homeostasis is yet unclear, although there seems growing evidence in favour of such a role (92).

5.3.3. Postsynaptic factors

ACh-stimulated opening of AChR channels allows for conductance of cations in a rather non-specific manner. Besides Na^+ influx and K^+ efflux, some Ca^{2+} fluxes inwards, which has been established to comprise ~4% of the total current in mice and ~7% in humans (15). Thus, the local subsynaptic Ca^{2+} concentration of the muscle fibre reflects the AChR density and might thus form part of the postsynaptic sensing mechanism leading to release of a retrograde factor which influences presynaptic ACh release. A similar role for postsynaptic Ca^{2+} has been proposed in plasticity of hippocampal synapses (93). If such a mechanism would be present at the NMJ, one would expect that in the case of the slow-channel AChR MG variant, the quantal content would be not or much less increased because more Ca^{2+} fluxes in than in MG with normal AChR kinetics. This was however not the case in our earlier study of NMJs in a biopsy of a patient and muscles of model mice with an autoimmune form of slow-channel AChR myasthenic syndrome; quantal contents were found to be considerably increased, to a similar extent as in typical MG (94).

CaMKII is an interesting candidate postsynaptic sensor because its enzymatic activity depends on Ca^{2+}. In *Drosophila* NMJs it has been shown that reducing postsynaptic CaMKII activity enhances presynaptic neurotransmitter release, while increasing its activity leads to reduction of quantal content (95). Our own earlier result that incubation of rat TIMG muscles with KN-62, an inhibitor of CaMKII, reduced rather than further increased the quantal content at NMJs is not compatible with these observations (58). A reason could be that KN-62 is less specific in inhibiting CaMKII than previously thought (96). Furthermore, the possibility exists that CaMKII is also involved in the *pre*synaptic compartment in the process of quantal content upregulation at the mammalian NMJ (97).

Dystrophin is a very large protein localized intracellularly at the skeletal muscle fibre sarcolemma where it connects actin to the dystrophin-associated glycoprotein complex. Most interestingly, it is present at increased density in the postsynaptic membrane of the NMJ. In *Drosophila*, muscle fibre dystrophin has been hypothesized to be involved in synaptic homeostasis (71). Postsynaptic deletion of dystrophin in *Drosophila* causes increased presynaptic neurotransmitter release at larval NMJs, suggesting that dystrophin normally suppresses release, possibly via an influence on retrograde trans-synaptic signalling mediated by the dystrophin glycoprotein complex (98). In our laboratory we have recently studied NMJ function of *mdx* mice (lacking dystrophin) as well as *mdx* mice with additional hetero- or homozygous *null*-mutation for utrophin. Utrophin is a dystrophin homologue which is also concentrated at the NMJ and is upregulated in absence of dystrophin. We observed that NMJs of all three mutant mice strains had, on average, ~40% smaller MEPP amplitudes compared to wild type, indicating loss of postsynaptic ACh sensitivity (20). A substantial increase (~50%) in mean quantal content as compared to wild-type was found in all strains. No statistically significant differences were found amongst *mdx*, *mdx/utrn*$^{+/-}$ and *mdx/utrn*$^{-/-}$ mice, indicating that the effect was primarily due to dystrophin deficiency and that utrophin is not of further influence. The increase was not equal at all NMJs; the quantal content inversely correlated with the postsynaptic ACh sensitivity (measured as MEPP amplitude) and was on wild type level at those NMJs which had MEPPs in the higher (i.e. wild type) amplitude range. Thus, a similar relationship between quantal content and MEPP amplitude was present as described above for NMJs of MG patients and animal models. This intact homeostatic adaptation of quantal content, correctly matched to the degree of ACh sensitivity reduction at single NMJs, indicates that dystrophin and utrophin are not crucially involved in the underlying mechanism at the mammalian NMJ, in contrast to the situation at the *Drosophila* NMJ.

Another very recently proposed postsynaptic factor is leucine-rich repeat kinase-2 (LRRK2). This kinase phosphorylates the ribosomal protein S15, thus enhancing protein synthesis, is expressed as orthologues in *Drosophila* as well as mammals, and thought to be involved in Ca^{2+} regulation (99-101). When LRRK2 was tissue-specifically knocked down in muscle cells of *Drosophila* larvae, the capacity of the NMJ to presynaptic upregulation of quantal content in response to (chronic) postsynaptic glutamate receptor reduction became impeded (102). Conversely, postsynaptic LRRK2 overexpression resulted by itself in an increase of presynaptic neurotransmitter release. The retrograde signalling factor associated with LRRK2 function has not yet been identified.

6. POSTSYNAPTIC ADAPTATION TO THE PRESYNAPTIC DEFECT AT NEUROMUSCULAR JUNCTIONS IN LAMBERT-EATON MYASTHENIC SYNDROME?

In view of the clear potential of synaptic plasticity at the NMJ it might be expected that not only presynaptic compensations to postsynaptic defects exist, but also the other way around, i.e. increases in postsynaptic neurotransmitter sensitivity in reaction to reduced presynaptic neurotransmitter release. Lambert-Eaton myasthenic syndrome (LEMS) is a rare presynaptic variant of myasthenia, in which >85% of patients has autoantibodies against the $Ca_v2.1$ type presynaptic Ca^{2+} channel (103). These autoantibodies cause a reduced presynaptic Ca^{2+} influx in response to the arrival of a motor neuronal impulse and thus cause reduced quantal content. This leads to subthreshold EPPs which underlie the muscle weakness. Whilst there is some evidence of compensatory presynaptic expression or unmasking of non-$Ca_v2.1$ type Ca^{2+} channels (104-106), no evidence so far exists for increased postsynaptic sensitivity for ACh, e.g. due to augmented AChR function or density. At NMJs of passive transfer models as well as of LEMS patient muscle biopsies, MEPP amplitudes are equal to those found in normal controls (105, 107)(J.J. Plomp unpublished observations). Similarly, at the *rolling Nagoya* mouse, which has a loss-of-function $Ca_v2.1$ mutation that leads to greatly reduced quantal content at the NMJ, no compensatory increase in MEPP amplitude was observed (108). Furthermore, at NMJs with *increased* quantal content, found in aged mice carrying a $Ca_v2.1$ gain-of-function *knock-in* mutation, no compensatory decrease in MEPP amplitude was observed (109). Thus, it seems that postsynaptic compensations in response to altered levels of presynaptic neurotransmitter release level generally do not occur at the NMJ. Perhaps the already extremely high density of ~10,000 AChRs per μm^2 at the postsynaptic membrane precludes any upregulation.

7. CLINICAL ADVANTAGE OF HOMEOSTATIC QUANTAL CONTENT UPREGULATION AT THE MYASTHENIC NEUROMUSCULAR JUNCTION?

The question arises as to whether the phenomenon of upregulation of ACh release at myasthenic NMJs is of clinical benefit. Most advantage is to be expected only when the reduction of AChRs is still intermediate. It is obvious that when the AChR reduction is extremely severe, even greatly increased ACh release will no longer produce EPP amplitudes above the firing threshold of muscle fibres, and muscle weakness will become more prominent. In addition, benefit is expected to be more outspoken at the start of muscle contractions rather than upon prolonged activity, because the homeostatic adaptation results in an initially enhanced ACh release level, but also in extra EPP rundown during high frequency use of the synapse, as described above. This might partially neutralize the beneficial effect during sustained use of the muscle. Interestingly, the symptoms of muscle weakness in MuSK MG patients, in which presynaptic compensatory increase of ACh release seems absent (25, 110), are often more severe and generalised than in most AChR MG patients, and more often lead to respiratory crises (111). It is tempting to suggest that this means that homeostatic ACh release upregulation confers some protection. However, it is clear that other reasons may also underlie, e.g. differences in the regional patterns of susceptibility of muscles for the different types of auto-antibodies (112).

Many commonly prescribed drugs are known or suspected to worsen muscle weakness in MG, sometimes without a clear explanation for these adverse effects (the University of Illinois College of Pharmacy lists as much as 17 common drug categories; https://pharmacy.uic.edu/departments/pharmacy-practice/centers-and-sections/drug-information-group/2014/2015-faqs/april-2015-faqs). Some or part of these effects may possibly be related to an influence on the homeostatic mechanism instead of directly affecting AChRs or the ACh release mechanism. In addition, polymorphisms in genes encoding relevant key factors in the homeostatic mechanism may render individual MG patients less well able to respond with an increase in ACh release at their myasthenic NMJs. It will be important to identify key post- and presynaptic factors as well as the retrograde signals at the NMJ that are involved in compensatory homeostatic upregulation of ACh release because these may form new therapeutic targets. Especially interesting may be diffusible retrograde signalling factors, which perhaps could be administered to specifically stimulate the motor nerve terminal to release extra ACh so that the myasthenic muscle weakness will be relieved.

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