Gangliosides and anti-ganglioside antibodies in neuromuscular synaptic function

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CHAPTER 4

Summary and general conclusion

Gangliosides form a group of sphingolipids that is much enriched in neuronal membranes, especially at synapses including the presynaptic membrane of the neuromuscular synapse. Therefore, they have been hypothesized to play important roles in neuronal function and in particular in neurotransmission. In this thesis the physiological and pathophysiological roles of gangliosides have been studied. Firstly, it was examined directly whether they play an important role in the release of neurotransmitter from the motor nerve terminal by studying synaptic transmission at NMJs of several types of ganglioside-deficient mice. It was found that both at young and older age, neurotransmitter release was not drastically impaired when subsets of the ganglioside family were lacking in knockout mice. Using electrophysiological methods, only subtle changes could be detected. Secondly, synaptic effects of antibodies against the gangliosides were studied at NMJs *in vitro*; in particular of antibodies directed at GM1 and at specific ganglioside-complexes. It was shown that these antibodies were able to interfere with neurotransmitter release in such a manner that block of neuromuscular transmission resulted, causing muscle paralysis. These effects were completely complement-dependent. Thirdly, an *in vivo* paralytic mouse model for anti-GQ1b ganglioside-induced peripheral neuropathy was developed. Finally, newly developed complement inhibitors were tested in the *in vitro* and *in vivo* neuropathy models. They were found to successfully prevent the neuropathophysiological effect of anti-ganglioside antibodies. In the sections below each of these themes will be discussed.

The role of gangliosides in NMJ neurotransmitter release

Gangliosides are part of the large family of glyscosphingolipids, which also includes the lacto-, globo-, and neolactoseries of lipids. This diverse group of glycosphingolipids is essential in life: mice that cannot express any of them, through a disrupted cGlcT gene, which encodes for glucosylceramide synthase, or a disrupted *Ugcg* gene, encoding for lactosylceramide (see figure 1.9), already die in the embryonic phase (Allende and Proia, 2002; Jennemann et al., 2005). When expression of solely the gangliosides is impaired (downstream of lactosylceramide), mice are viable (Yamashita et al., 2005), which suggests that gangliosides are redundant for at least early developmental functions. It was found that gangliosides play a role in many physiological processes in the human body, some of which are specific neurological functions like maintenance of the nervous system and axonal regeneration (Yamashita et al., 2005; Ribeiro-Resende et al., 2007). Gangliosides are especially abundant in the presynaptic membrane and co-localize with the exocytotic machinery (Chamberlain et al., 2001; Lang et al., 2001), suggesting they play a role in transmitter release. This section focuses on the results from our studies on ganglioside functioning in neurotransmitter release as described in this thesis.

Gangliosides and the NMJ

Ando and colleagues (1998) reported that gangliosides can act as modulators of synaptic functions: exogenous addition of ganglioside GQ1b or GM1 enhanced ACh release upon stimulation in synaptosomes. It was also shown that the gangliosides are especially enriched in presynaptic membranes (Hansson et al., 1977; Ledeen et al., 1993) and pathophysiological effects on neurotransmitter release (i.e. alterations in synaptic activity) induced by anti-ganglioside antibodies present at the NMJ, were

shown in several functional studies (Santafé et al., 2008; Buchwald et al., 2007; Nakatani et al., 2007b; Nakatani et al., 2009; Buchwald et al., 2002; Ortiz et al., 2001; Roberts et al., 1994; Plomp et al., 1999; Goodyear et al., 1999). These observations suggest that gangliosides play a specific and possibly essential role in ACh release at the motor nerve terminal. In a previous study with mice lacking the complete set of complex gangliosides (GM2s-KO mice), the first attempt to elucidate electrophysiological effects from an altered ganglioside composition in the NMJ was performed (Bullens et al., 2002). It revealed only subtle changes in transmitter release properties, like a reduced transmitter release at 17 °C, but no (sub)clinical deficits could be found. Here, other types of knockout mice were used for examination of the role of gangliosides in the release of neurotransmitter in the NMJ, i.e. mice expressing only the O- and a-series (GD3s-KO mice), or solely GM3 (i.e. dKO mice that have impaired GD3synthase and GM2synthase genes; Figure 1.9). However, these studies did not reveal crucial importance of any single ganglioside for transmitter release in the various ganglioside profiles tested. Even when *in vitro* all sialic acids were removed (by treating dKO mouse diaphragms with the enzyme neuraminidase from *Clostridium perfringens*), no major changes in neurotransmitter release occurred. Thus, based on these results, gangliosides seem to have a modulating function in NMJ neurotransmitter release rather than a crucial influence. This is quite unexpected in view of the presence of such an elaborate system of gangliosides in the neuronal membrane, especially at synapses. This presence was clearly indicated in the experiments with anti-ganglioside antibody incubations (or injections in case of the *in vivo* model for MFS) by presynaptic binding of these antibodies, as directly shown with immunofluorescence microscopy, as well as indirectly by the neuropatho-physiological effects observed in the electrophysiological studies. It might be, though, that the different gangliosides are well able to compensate each other's functions, which makes it difficult to show an absolute requirement for gangliosides in transmitter release. But, while the experiments with disrupted GM3, the last remaining ganglioside at dKO NMJs, indeed showed some degree of such compensation, they also clearly demonstrated that gangliosides have no fundamental function in transmitter release. Therefore, it has to be concluded that, at least at the NMJ, gangliosides have a modest role in synaptic transmission.

Subtle changes in high frequency neurotransmitter release due to alterations in ganglioside subset expression

Although we did not encounter clinical impairment of NMJ transmitter release in the muscles of ganglioside knockout mice tested, we did find some subtle changes. In dKO NMJs at high frequency nerve stimulation an increase in the physiological rundown of ACh release was observed compared to the control mice. The extent of this phenomenon was modest, the steady state rundown-level being only 5-7% lower than in wildtype controls. This more pronounced rundown was most obvious at frequencies of 30-70 Hz and was not seen at 3 Hz. In dKO tissues lacking all gangliosides due to neuraminidase degradation of GM3, rundown became even more pronounced (~20% lower than was seen in wildtype controls). When studied in older mice, where possible maintenance functions of gangliosides might become more important, we also found extra rundown for GM2s-KO mice, but not for GD3s-KO mice (aged dKOs could not be tested due to early death of this strain). One factor in sustained transmitter release is the pool size of readily releasable synaptic vesicles. It seems unlikely, though, that this pool is influenced by gangliosides, since application of hypertonic medium showed equal elevated levels of spontaneous ACh release, an

indirect measure of pool size, for all genotypes tested. Since GD3s-KO NMJs did not show the pronounced transmitter release rundown effect, we hypothesized that the O- and a-series gangliosides might be involved in high frequency transmitter release. The evoked release of ACh is crucially dependent on $Ca_v2.1$ Ca^{2+}-channels (Urbano et al., 2008; Uchitel et al., 1992). Experimental results suggest that there is a relationship between gangliosides and these Ca^{2+}-channels (Ortiz et al., 2001; Nakatani et al., 2009) and it was also shown that addition of the a-series gangliosides increased evoked ACh release from synaptosomes (Tanaka et al., 1997). There might be a role in this activity for the sialic acid residues of the gangliosides that protrude into the extracellular space and can bind Ca^{2+} (Hayashi et al., 1984; Rahmann et al., 1990). The high affinity for Ca^{2+} may concentrate the ions in the vicinity of the Ca^{2+}-channels and facilitate the transport through the channels. These interactions might modulate Ca^{2+}-dependent processes of synaptic transmission with an effect that is subtle, and only becomes apparent at intensive high frequency use of the synapse.

Temperature stabilizing effects of gangliosides

Gangliosides are hypothesized to have a role in thermal adaptation of neuronal membranes (Rahmann et al., 1998). It was shown in fish that the polarity (i.e. number of sialylated fractions) of the ganglioside composition is modified specifically in neuronal membranes after (long-term) thermal adaptation, whereas in liver and muscle, adaptations of other lipids were detected (e.g. cholesterol in muscle). This reasonably suggests that the Ca^{2+}-binding property of the membrane is also thermosensitive, as pure sialic acid has the highest affinity for Ca^{2+} (Rahmann et al., 1998). Furthermore, GQ1b and GD1a were found to reduce ion channel-conductance in a thermosensitive manner as well, by affecting the fluidity of the membranes in the areas surrounding the channels (Kappel et al., 2000). Therefore, with the study of ganglioside functioning in transmitter release here, different bath temperatures were tested in order to explore the thermal influence on transmitter release in the presence of the different ganglioside subsets in the presynaptic membrane. It was already found in GM2s-KO mice that at 17 °C both evoked and spontaneous transmitter release were reduced, compared to wildtype controls at the same temperature (Bullens et al., 2002). In this thesis we also encountered temperature-dependent alterations. In short: at 17 °C, NMJs in young GD3s-KO mice showed a significant increase in the amount of ACh that was released spontaneous or evoked, compared to wildtype. At 35 °C an increase was seen for evoked release only. This tendency of increased release of the amount of ACh was also observed in the aged GD3s-KO mice, though at all temperatures tested (17, 20, 25, 30, and 35 °C). Furthermore, there was a slight tendency of an increase in evoked ACh release (calculated as quantal content) with temperature in young dKO and GD3s-KO mice and at 35 °C the evoked release in GD3s-KO NMJs was even ~40% higher compared to wildtype NMJs. This feature was not observed in the aged mice that were measured. In aged GM2s-KO and GD3s-KO NMJs, however, spontaneous ACh release (measured as MEPP frequency) was decreased compared to wildtype at 17 °C. These findings indeed indicate a temperature-dependent role for gangliosides in neurotransmitter release. But apart from the 40% increase in transmitter release at 35 °C in GD3s-KO NMJs, the effects are rather modest and are likely not physiologically relevant.

Ca^{2+}-dependent alterations

In neuronal cells from GM2s-KO mice, lacking complex gangliosides, impaired Ca^{2+}-regulating properties were found (Wu et al., 2004). In this thesis, this role of

gangliosides in Ca^{2+}-regulation was further explored. Mouse muscle-nerve preparations were exposed to low or high (non-physiological) Ca^{2+}-concentrations in the bath-applied medium. Using these extreme conditions we tried to elucidate a possible Ca^{2+}-dependent effect of gangliosides on neurotransmitter release at the NMJ. As expected, the evoked and spontaneous release of ACh was steeply dependent on the extracellular Ca^{2+}-concentration. However, we did not observe differences in transmitter release parameters between the NMJs of the diverse ganglioside knockout mice, except for aged GD3s-KO mice NMJs. At 0.2 mM Ca^{2+}-concentration we found increased ACh release compared to wildtype and GM2s-KO NMJs. The Ca^{2+}-buffering capacity of the progressively increased density of GM1 ganglioside in the aged GD3s-KO mice might be responsible for this effect (Wu et al., 2004; Wu et al., 2007; Okada et al., 2002; Handa et al., 2005).

Alterations in transmitter release parameters in aged mice

Neuronal ganglioside compositions vary throughout life and gangliosides appear to become increasingly important upon ageing (Svennerholm et al., 1989; Yamamoto et al., 2008). In this thesis two studies on neurotransmitter release parameters in ganglioside knockout mice are described, one of which performed in NMJs of mice at young age (6-13 weeks) and the other in NMJs of old mice (>9 months). When comparing the results of the two studies, we find age-related changes in neuromuscular transmission. First of all, we encountered lower amplitudes of both EPPs and MEPPs in the aged mice compared to young ones. This is not due to alterations in ganglioside composition, because it was also seen in young versus old wildtype controls. Most likely this is related to the fact that young mice have muscle fibres which are smaller in diameter and have a higher input resistance than the muscle fibres of old mice. MEPP amplitude is proportional to the muscle fibre resistance (Harris and Ribchester, 1979). The MEPP frequency however, did show an age- *and* ganglioside-related alteration. MEPP frequency in NMJs of aged GD3s-KO mice was 43% higher than in aged wildtype mice and on average 46% higher than in all the young mice, including the young GD3s-KO mouse. The GD3s-KO mice have the O- and a-series gangliosides upregulated in their membranes, one of which is GM1. GM1 has been shown to influence Ca^{2+}-buffering and membrane flux and, being upregulated, this ganglioside may therefore account for the higher MEPP frequency (Wu et al., 2004; Wu et al., 2007). Also, gangliosides contain negatively charged sialic acid residues that contribute to the membrane surface charge. In the vicinity of Ca^{2+}-channels, an increase in density of O- and a-series gangliosides in the presynaptic membrane may therefore directly influence the kinetics and function of these ion channels (Wang et al., 1999b; Ledeen and Wu, 2006a).

Furthermore, at aged GD3s-KO NMJs we observed decreased rise times and halfwidths of both EPPs and MEPPs, not previously seen at NMJs of young GD3s-KOs or any of the other ganglioside-deficient mice. A presynaptic explanation for these kinetic changes does not seem likely, as gangliosides were reported to lack effect on the synthesis of ACh (Tanaka et al., 1997). Rather, postsynaptic gangliosides might modulate the behaviour of ACh receptors on the muscle membrane. Gangliosides co-localize with these receptors (Pato et al., 2008; Marcheselli et al., 1993) and in the membranes from *Torpedo marmorata* they were found to interact (Mantipragada et al., 2003). Alternatively, progressive postsynaptic a-series upregulation might have influenced the passive electrical characteristics of the muscle fibre membrane (i.e. capacitance and resistance). However, these parameters were not directly determined. Again, a progressive increase of the a-series gangliosides with

age in GD3s-KO mice (as may occur in muscle membranes alike in neuronal membranes), could lead to these kinetic alterations that were not visible in the young GD3s-KO mice or in any of the other genotypic mice. It is an interesting feature that deserves more study.

Interpretation and extrapolation of mouse studies

Differences in effects of ganglioside deficiencies in mice and humans

For this thesis three types of ganglioside knockout mice were used: GM2s-KO mice, which lack all complex gangliosides, GD3s-KO mice, which only express the O- and a-series gangliosides, and the dKO mice that can only express ganglioside GM3. Whether the phenotypical features of these mice (as described in chapter 1) are due to the lack of particular gangliosides or to an *overexpression* of the remaining ones is not clear. Accumulation of ganglioside GD3, for example, can alter mitochondrial function and induce cell death in a caspase-dependent manner (De Maria et al., 1997), and also GM3 was found to be a modulator of *in vivo* neuronal cell death (Sohn et al., 2006): overexpression of these gangliosides may account for (part of) the pathophysiological features that are seen in aged GM2s-KO mice. On the other hand, the pathophysiological phenotype of these mice may also be due to an impaired nerve maintenance function for which some of the complex gangliosides are required (Vyas et al., 2002; Yamashita et al., 2002; Cao et al., 2007; Yang et al., 1996).

Simpson and colleagues (2004) identified a human autosomal recessive infantile-onset symptomatic epilepsy syndrome associated with developmental stagnation and blindness. Patients with this syndrome had a nonsense mutation in the SIAT9 gene, which results in the premature termination of the GM3synthase enzyme. Individuals indeed lacked GM3 and its biosynthetic derivatives, and had an increase in their plasma of lactocylceramide and its alternative derivatives. Primary cultures of skin fibroblasts of three patients homozygous for this mutation were established for research. The fibroblasts exhibited a large reduction (93%) in cellular ganglioside content, which was not compensated for by activation of an alternate pathway of ganglioside synthesis. In fibroblasts from GM3s-KO mice, however, compensation did occur: the O-gangliosides were found to be expressed in the fibroblasts, which do not normally express these gangliosides (Shevchuk et al., 2007; Liu et al., 2008). Unlike the patients described by Simpson and colleagues (2004), the GM3s-KO mice do not show seizure activity (Yamashita et al., 2003). However, mice that have a mutated SIAT8a gene (encoding GD3synthase) *and* a mutated Galgt1 gene (encoding for GM2synthase) do show susceptibility for lethal audiogenic seizures (depending on the strain) (Kawai et al., 2001), which is alike the epilepsy that is seen in humans with a deficit in the SIAT9 gene. It can be hypothesized that basically the function of the gangliosides is not different in mice from humans. However, a deficit in the ganglioside biosynthetic pathway apparently can be compensated for in mice and not in humans (as is seen in the cultured fibroblasts). This can account for (part of) the phenotypic differences. Until now no other human patients are described that lack subsets of gangliosides, therefore no further conclusions can be made on this issue.

NMJ dysfunction in human GBS patients

About half of the GBS patients and over 90% of the MFS patients were found to have anti-ganglioside antibodies in their acute phase serum. (Willison et al., 1993; Hafer-

Macko et al., 1996b; Lange et al., 2006; Van Doorn et al., 2008). Antibody titres are highest in the acute phase and decrease with clinical improvement, suggesting a pathogenic role. Several studies using single fibre electromyography or repetitive nerve stimulation in MFS patients suggest that the weakness of the ocular muscles is due to a presynaptic neuromuscular transmission defect, which was found to coincide with the elevated antibody titre of anti-QG1b in the patient's serum (Lange et al., 2006; Lo et al., 2004; Lo et al., 2006; Sartucci et al., 2005). This rightly fits with the pathological features in our mouse models, in which presynaptic binding of anti-ganglioside antibodies at the mouse NMJ leads to impaired neuromuscular transmission and structural damage of the motor nerve terminal in a complement-mediated fashion. To my knowledge there are no reports of motor endplate biopsies taken from MFS patients, in which complement deposition has been studied; in post mortem studies of GBS patients, however, the deposition of complement at the NMJ has been reported (Koski et al., 1987; Hafer-Macko et al., 1996a; Hafer-Macko et al., 1996b) and it was also found that GBS patients have an elevated level of the complement components C3a and C5a in their cerebrospinal fluid (Hartung et al., 1987). Therefore, the mechanism of action of the anti-ganglioside antibodies as seen in our *in vitro* and *in vivo* mouse models shows similarities to the human pathological situation in MFS and GBS. Yet, it should be noted that anti-ganglioside antibodies are not exclusively encountered at presynaptic nerve membranes, but were also found to bind gangliosides in the membranes of perisynaptic Schwann cells in mice (O'Hanlon et al., 2001) and in patients with the AMAN variant of GBS the target sites for antibodies were found to be the axolemma and the nodes of Ranvier (Hafer-Macko et al., 1996b). The more proximal nerve sites (including the nodes of Ranvier), however, showed little penetration and binding of antibodies in mice during a 6 h time window, most probably due to the protection of the blood nerve barrier (Paparounas et al., 1999); this makes the NMJ, which lies outside the blood nerve barrier, a more suitable model site for studying the pathological mechanisms of GBS.

The immune system

When studying autoimmune neuropathies, also the immune system must be taken into account. Complement activity, as part of the innate immune system, differs between humans and other mammals, although there are many similar factors. It was reported that common laboratory mouse strains have much lower complement activity than humans (Rice, 1950; Ong and Mattes., 1989), and the anti-GQ1b IgM that we used in our *in vivo* mouse model for MFS was not able to activate the mouse complement, although this antibody was a very potent activator of the human complement. Important when using mouse models is the finding that also between mice strains the immune system functioning can differ. This was for example shown for antibody class switching of mature B cells that differed among three common strains of mice (Kaminsky and Stavnezer, 2007). Also, it was thought that BUB mice expressed complement that was far more active than was seen in other laboratory mice (although still not as active as in human) and three strains that were (recently) derived from the wild even had complement levels as high as rabbits and rats (Ong and Mattes, 1989; Ong et al., 1992). Adding to the complexity, more recently (Osmers et al., 2006) it was reported that complement activity in most mouse inbred strains is similar, but that the result depends on the type of assay that is used. For instance, BUB mouse serum shows increased performance in hemolytic assays compared to other strains, but no elevated activity in opsonization experiments using bacteria. Therefore it should be realized that experimental complement-mediated results depend on the type of assay

or the animal model that is used and that general conclusions about the relevance to human conditions are not easy to make.

***In vivo* mouse model for MFS**

In this thesis we developed an *in vivo* mouse model for MFS that may be very useful in upcoming studies of newly developed complement inhibitors, neuroprotective agents or other experimental drugs aimed at limiting the nadir and/or facilitating recovery of the paralytic symptoms of GBS. In short, the model was generated as follows: the mouse received an intraperitoneal injection with a purified monoclonal anti-GQ1b ganglioside antibody. After an overnight incubation period, a second injection with normal human serum as a source of complement was given. After some hours (depending on the antibody dose) the mouse developed breathing problems and became lethargic. Breathing and behavioural parameters were recorded before and during the experiment. At the end of the experiment (~6 h after the source of complement was injected), the diaphragm with phrenic nerve attached was dissected and electrophysiological measurements were performed. While creating this model, several attempts were made to induce neuromuscular paralysis without adding normal human serum as heterologous complement source. However, we were not able to achieve this. Apparently the low activity of endogenous mouse complement prevented development of a deleterious effect, as explained above. Even the use of knockout mice that lacked particular complement regulation factors, facilitating complement activation on mouse self membranes, did not yield a paralytic model with a clear neuropathology. Therefore exogenous complement was inevitable in the current model. This raised questions about the relevance of the model, which also has some drawbacks since it involves an extra injection in the abdomen (for the complement addition) and the complement concentration in the abdomen at that point is rather high compared to other sites in the body, due to the localized injection of the complement. But it turned out that the addition of an exogenous source of human complement made the model very practical when testing compounds that specifically inhibit *human* complement factors such as the C5-inhibiting monoclonal antibody eculizumab. Therefore, the combined model of passive immunization with mouse monoclonal antibodies against the GQ1b ganglioside in combination with normal human serum as complement source forms a rather straightforward model for MFS that can be applied in future drug studies.

Gangliosides and their native micro-environment

Antibody binding

The experimental work described in this thesis demonstrated an interesting discrepancy between the actions of anti-ganglioside antibodies in ELISA and in live neuronal tissue. The study on anti-GM1 antibodies showed that differences in affinity of patient sera or antibodies in ELISA tests were not well-predicting their effect at the mouse NMJ in live tissue. Apparently, complications due to ganglioside configuration in the living membrane exist. Gangliosides may interact with each other or with other membrane components of the lipid microdomains in which they are present (Hakomori, 2003). Like, for example, gangliosides and cholesterol co-localize in microdomains in the vicinity of the neurotransmitter release machinery (Simons and Ikonen, 1997). According to Cantu and colleagues (2009) gangliosides are likely to play their physical roles in a three-dimensional manner through curvature, metamorphism (i.e. displaying multiple space filling geometries), cooperativity and demixing (both in presence and absence of cholesterol). An interesting example of

this is seen in a study by Terrell and colleagues (2008), who found that liposome membrane fluidity played a major role in cholera toxin B-subunit binding. GM1 is a natural ligand for the B-subunit, and after their binding, cholera toxin A subunit can enter the cell (being the biologically active protein). When liposomes were prepared with cholesterol added in, the binding ability decreased. Presumably, cholesterol would act to make the liposomes, which are quite rigid at room temperature, more fluidic. This increased fluidity of the membrane may affect the ability of GM1 to allow binding of cholera toxin. This example stresses the importance of the local membrane properties in determining the binding potency of ligands to gangliosides. Thus, it may well be that binding specificities of antibodies, as defined with ELISA assays, do not faithfully reflect the binding properties *in vivo*, because gangliosides in ELISA are not embedded in a biological membrane. Therefore, one need to be careful to draw a conclusion based on a single type of assay. On top of that, the accessibility of gangliosides may not only vary between assay-environment, but also between sites within the human body. While sensory and motor nerves express similar quantities of GD1a and GM1 (Gong et al., 2002), the AMAN-associated anti-GD1a/GM1 antibodies only affect motor neurons. Lopez and colleagues (2008) studied the fine specificity of ganglioside orientation and exposure in the two nerve types and found a differential expression of a critical GD1a epitope for antibody binding. Also O'Hanlon and colleagues (1998) showed that immunoreactive epitopes for anti-GM1 antibodies, are not confined to the motor system, but are ubiquitous within the human nervous system. They state that if anti-GM1 antibodies are central to the pathogenesis of AMAN, then "a simplistic present or absent approach to epitope distribution at clinically affected or unaffected sites is not sufficient to account for their effects". In the experimental models used in this thesis, most studies were performed in mouse phrenic nerve-diaphragm preparations: following the above, it remains unclear whether findings can be directly applied to NMJs in other muscles. Ganglioside GQ1b appears to be prominently expressed in the motor nerve terminals of the mouse phrenic nerves, but this is not confirmed for human phrenic nerves. The breathing difficulties in the mouse model following anti-GQ1b antibody immunization may be exaggerated due to the intraperitoneal injection, maximally exposing the diaphragm. Breathing difficulties are not a general feature in MFS patients (Mori et al., 2001). However, another study (Kaida et al., 2004a) suggests that GBS patients with anti-GQ1b antibodies more often require mechanical ventilation, although responsibility of a possible anti-GT1a antibody activity could not be excluded. Furthermore, in MFS patients with anti-GQ1b antibodies present in their serum no affected limb muscles were reported (Kuwabara et al., 2007); in the mouse model, however, we also found anti-GQ1b antibody binding at limb NMJs. So, pathological activity of anti-ganglioside antibodies is likely determined by both the specificity of the ganglioside composition in the membrane and the interaction of these gangliosides with their surroundings.

Acetylcholine release and recycling

As mentioned above, gangliosides and cholesterol co-localize in membrane lipid rafts near the neurotransmitter release machinery. An important factor for ACh release at the NMJ might be found in the presence of cholesterol as a prominent component in the presynaptic membrane. Cholesterol has been proposed to function as a spatial organizer of synaptic vesicle recycling. Wasser and colleagues (2007) reported observations that suggest that synaptic cholesterol is a crucial component of the machinery and prevents excessive spontaneous fusions of synaptic vesicles. Therefore

they state that proper maintenance of synaptic function requires tight regulation of cholesterol levels in the membrane. Removal of cholesterol is likely to increase membrane fluidity and this may promote vesicle fusion, as was seen at crayfish NMJs (Zamir and Charlton, 2006). It has been reported that cholesterol is regulated and metabolized in a manner that is entirely independent of the metabolism of complex gangliosides. However, in dKO mice small elevations of cholesterol levels were found (Li et al., 2008). Since cholesterol and gangliosides are present in the same lipid rafts, it might be possible that deletion of particular gangliosides interferes with cholesterol functioning at the presynaptic terminal. Therefore, if ganglioside deletion has an indirect effect on synaptic vesicle (re)cycling at the NMJ, long-duration stimulation protocols (lasting for minutes, rather than seconds, as studied in this thesis) might reveal such a putative role.

Exogenous addition of gangliosides

Another interesting point of study may be the effect of exogenous addition of specific gangliosides to the NMJ. The combination of murine ganglioside knockout tissue with the exogenous application of gangliosides should make it possible to obtain every single combination of gangliosides in the presynaptic membrane. Valaperta and colleagues (2007) reported that gangliosides added to culture medium under proper conditions are taken up by the cells and are inserted as monomers in the membrane bilayer, becoming virtually indistinguishable from the endogenous compounds. Thus, treatment with exogenous gangliosides is an efficient way to change the ganglioside content of the plasma membrane. Thereafter it would be even more interesting to obtain living mice with certain ganglioside compositions not only based on gene knockout, but also on addition of gangliosides via for example the food. It has already been shown that dietary gangliosides can change ganglioside compositions in brain cell membranes in living rats (Park et al., 2005). If there is a particular combination of gangliosides that induces vulnerability for certain types of GBS, such a manipulation of ganglioside expression might be a way to reveal this. However caution must be taken as there might need to be drawn a distinction “between intrinsic functions of gangliosides as naturally expressed by the cell, and activities created by application of exogenous ganglioside(s) that may or may not reflect the natural function” (Ledeen and Wu, 2002). This can be complex, as was for example shown by Wang and colleagues (2006), describing a feedback mechanism for sialic acid expression by the enzyme UDP-N-acetylglucosamine-2-epimerase/N-acetylmannosamine kinase (GNE). GNE catalyzes the rate limiting step in ganglioside sialic acid biosynthesis, but is itself regulated by gangliosides GM3 and GD3, which demonstrates a reciprocating feedback mechanism where gangliosides regulate an upstream biosynthetic enzyme. And as it turned out, also exogenous GM3 and GD3 were able to change the expression of GNE, meaning that addition of gangliosides can lead to more alterations than solely a higher membrane density.

Clinical considerations

Complement inhibition in neuropathies

Current treatment of GBS is not optimal yet. For one part this is because the aetiology of the disease is not completely elucidated and for another part because the onset of the disease is so acute. Generally, this acute phase is followed by a long recovery

period. To make this period shorter and easier, accurate action already in the acute phase is required. It is likely that the complement cascade is involved in GBS neuropathy (Hartung et al., 1987; Halstead et al., 2004). Post mortem studies of fatal cases of AMAN and AIDP revealed activation and binding of complement at motor nerves (Hafer-Macko et al., 1996a; Hafer-Macko et al., 1996b), and cytotoxicity assays demonstrated that neuronal cell lysis caused by serum from GBS patients is complement dependent (Zhang et al., 2004). In this thesis the mechanism of injury in experimental MFS/GBS is inhibited by the application of complement inhibitors: the monoclonal antibody eculizumab and the recombinant tick factor rEV576. When applied in the MFS mouse model, both therapeutics prevented splicing of component C5 and thereby the initial phase of the formation of membrane attack complex (MAC) as a final step in the complement cascade, which resulted in preservation of neuronal membrane integrity and neuromuscular transmission. Of outmost importance for these complement inhibitors to be effective in GBS patients will be administration early in the course of the disease. The deterioration in GBS happens within the first four weeks, when MAC is disintegrating nerve membranes and macrophages are attracted by the complement components, causing neuronal and glial injury, and nerves are degenerating (Hughes and Cornblath, 2005). When this deterioration phase comes to an end, no more injury will be initiated and regeneration will be the main process. This means that the effectiveness of the complement inhibitors will most probably be highest in the initial phase. Obviously, when the complement activity is inhibited early, less neuronal damage will occur. Complete nerve protection will not be possible, because the clinical presentation of the disease will almost certainly be preceded by complement activation. However, swift reduction of severity of the peak symptoms of the disease and amelioration of nerve injury is very desirable, because this likely positively influences long-term outcome. The complement inhibitors are a promising class of therapeutics for autoimmune neuropathies like GBS, but also for other complement-mediated autoimmune diseases like systemic lupus erythematosus and myasthenia gravis. For paroxysmal nocturnal haemoglobinuria (PNH), a genetic disease in which complement-mediated lysis of erythrocytes occurs, the complement inhibitor eculizumab has already been proven effective in human clinical trials (Hillmen et al., 2004; Hillmen et al., 2006) and since 2007 it has been approved by the Food and Drug Administration in the USA for the treatment of PNH patients (Brodsky, 2009). Clinical trials for eculizumab with autoimmune neuropathy patients are underway. The next experimental step could be to define where in the time path of the development of symptoms in the MFS/GBS mouse model, application of complement inhibitors are most effective and supportive for (long-term) protection and/or regeneration of the affected nerves.

Neuroprotective therapeutics

In addition to the complement system there are more candidate therapeutic targets in anti-ganglioside antibody-mediated neuropathies. For example, inhibition of the cytoplasmic protease calpain was found to protect against axonal degeneration. Calpain is activated via Ca^{2+} and elevated levels can impair neuromuscular transmission by disrupting motor axons. Several studies in animal models have shown that treatment with calpain inhibitors in (experimental) neuropathies improves neuronal survival (O'Hanlon et al., 2003; Kieran and Greensmith, 2004; Groshong et al., 2007). To my knowledge, calpain inhibitors are not yet applied in human patients, but they have been applied safely and successfully in animal models of neurodegeneration (Badalamente and Stracher, 2000). Another option for therapeutics

may be found in axonal protection by blocking the well-known inhibitory effects of myelin-associated glycoprotein (MAG) on neurite outgrowth in regenerating tissue, but also in exploitation of recently found beneficial effects of MAG, as were reported by Nguyen and colleagues (2009): in normal healthy tissue MAG supports axonal stability and survival by discouraging either longitudinal or collateral outgrowth, and by promoting maximal radial calibre of the axon. Enhancement of his feature could play a positive role when degeneration should be prevented. In an animal model for multiple sclerosis axonal loss was 52% more severe in mice that lacked MAG. This shows therapeutic possibilities that deserve further exploration.

Anti-ganglioside complex antibodies in GBS and MFS patients

It was first described by Kaida and colleagues (2004b) that not only single gangliosides can serve as antibody targets, but also complexes of two different types of gangliosides can together form a new, recognizable epitope for the anti-ganglioside antibodies. This implies that even in the ~40% of GBS patients in which no "classic" anti-ganglioside antibodies can be detected, antibodies against complexes might be present. Since these novel antibodies were reported, sera from patients have been screened and several antibodies against different complexes have been found. Anti-GM1/GalNac-GD1a complex antibodies are considered to be a target antigen in pure motor forms of GBS (Kaida et al., 2008b). Antibodies against GD1a/GD1b and GD1b/GT1b complexes were found to be associated with severe GBS requiring artificial ventilation (Kusunoki et al., 2007). Also in MFS patients, antibodies against GM1/GQ1b and/or GD1a/GQ1b complexes were detected (Kaida et al., 2006; Kusunoki et al., 2007). In addition, antibodies against ganglioside complexes (GM1/GD1a and GQ1b/GD1a) were shown to cross-react with lipooligosaccharides-epitopes on the membrane of *C. jejuni* (Kuijf et al., 2007), indicating again the possibility of molecular mimicry as the initiator of GBS. In this thesis we investigated 17 GBS sera with anti-GM1/GD1a complex activity and 10 GBS sera with anti-GM1/GQ1b complex activity, for their ability to induce complement-mediated neuropathophysiological effects at mouse NMJs. The effects of anti-GM1/GD1a sera were highly variable: after addition of a source of complement, 8 out of 17 sera induced increased spontaneous transmitter release. This effect was exclusively seen in NMJs of GD3s-KO mice (which have upregulated GM1 and GD1a), indicating the requirement of a high level of antigen density. The results were not unambiguous, though, because many sera were not antibody-complex specific, but also had some remaining activity against one or both of the individual gangliosides, which complicates analysis. Therefore, further study is required to unravel the exact neuropathic actions of this special antibody category. However, the discovery of these new antibodies may already be very helpful for aetiological understanding of GBS and it enhances the value of anti-ganglioside antibodies as diagnostic markers.

Ganglioside profile as a predisposing factor for GBS

It remains unclear why most people do not get GBS, but a small group does. In this thesis it is described that ganglioside knockout mice that have subsets of gangliosides upregulated are more vulnerable for antibodies directed against these particular gangliosides. For example, synapses from GD3s-KO mice did not exhibit pathophysiological effects when a particular anti-ganglioside antibody directed against GM1 was applied, but GD3s-KO mice synapses that were treated with neuraminidase (increasing the membrane density and absolute amount of GM1) showed enhanced vulnerability for development of an increase in spontaneous

transmitter release and asynchronous muscle fibre twitching after incubation with this same antibody (in combination with a source of complement). This indicates that high density of antigenic ganglioside may form a predisposing factor in GBS. Hence, conditions that temporarily or locally result in increased ganglioside level may predispose to GBS. An example of variable ganglioside expression was encountered in a study by Kato and colleagues (2008) who found an increase of endogenous hippocampal GQ1b following several kindled-seizures in a mouse model for human temporal lobe epilepsy. This is an indication that ganglioside expression can temporarily be altered by factors other than normal growth and development, which might enhance vulnerability to particular anti-ganglioside antibodies.

Conclusion

On the basis of the synaptic studies performed at various ganglioside-deficient mice, it can be concluded that gangliosides are not crucially involved in neurotransmitter release at the NMJ. Rather, gangliosides seem to play a role in temperature- and use-dependent fine-tuning of transmitter output. Given that the NMJ is considered a model synapse, it may well be that also at central synapses gangliosides only play marginal roles in synapse function. On the other hand, subtle changes may have profound effects on synaptic integration in central neurons and may thus considerably affect neuronal network function. The absence of an absolute role of gangliosides in synaptic transmission is surprising in view of the existence of such an elaborate ganglioside family, the enriched synaptic presence, and older studies showing synaptic effects of bath-applied gangliosides. It may be that gangliosides are more important in non-standard conditions, e.g. regeneration, extremely intense use, or in certain developmental stages. Or maybe the conclusion should be drawn that with all the present knowledge there seems to be no direct role for any of the gangliosides in the process of neurotransmitter release at the motor nerve terminal. In the end, gangliosides are not a universal feature of presynaptic motor nerve terminals, as *Drosophila melanogaster* has functional neuromuscular synapses, but does not express gangliosides (Roth et al., 1992; Chen et al., 2007a).
This thesis furthermore describes the protective properties of complement C5 inhibitors, eculizumab and rEV576, against the deleterious effects of anti-GQ1b antibodies and complement at the mouse NMJ. They were found to be very effective inhibitors of the pathophysiological effects in both the *in vitro* and the *in vivo* model for MFS. These studies indicate that complement inhibitors can restrict experimental neuropathophysiological damage, which suggests that these compounds may be clinically useful in limiting peak symptoms and speeding up recovery of GBS and other neuroimmunological disorders.