

Gangliosides and anti-ganglioside antibodies in neuromuscular synaptic function

ISBN: 978-90-8891-133-0

Uitgeverij BOXPress

Printed by: Proefschriftmaken.nl / Printyourthesis.com

Gangliosides and anti-ganglioside antibodies in neuromuscular synaptic function

Proefschrift

ter verkrijging van

de graad van Doctor aan de Universiteit Leiden,

op gezag van de Rector Magnificus prof. mr. P.F. van der Heijden,

volgens besluit van het College voor Promoties

te verdedigen op woensdag 20 januari 2010

klokke 15.00 uur

door

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geboren te Leiden in 1979

Promotiecommissie

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The studies described in this thesis were supported by the Prinses Beatrix Fonds (#MAR04-0213).

**Putting on the spectacles of science
in expectation of finding an answer to everything looked at
signifies inner blindness.**

-- Frank J. Dobie

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CHAPTER 1

General introduction

Preface

In the 1930's Ernst Klenk discovered new cell membrane elements in the brain of a patient who died from Tay-Sachs disease, a disorder characterized by progressive loss of mental abilities that already start at very young age. First Klenk called the elements substance X, but later, when he noticed that it concerned a mixture of compounds, he changed it into "gangliosides", believing that they were preferentially localized in the ganglia of the brain (Klenk, 1942; Debuch, 1973). Despite the enthusiasm of several scientists, it was not before 1963 that the biochemical structure of the first ganglioside was elucidated. When the whole family of gangliosides became known, proper naming for every separate ganglioside was needed. Therefore in 1964 a nomenclature for the different gangliosides was proposed by Lars Svennerholm in the *Journal of Lipid Research* and this is still used today (Svennerholm, 1964). In 1994 a Nobel symposium was organized, devoted to gangliosides only. Nowadays not only the structures of this diverse class of lipids are known, but also different kinds of transgenic mice exist for research objectives, lacking particular combinations of gangliosides. Despite the knowledge of the molecular properties of the different gangliosides, the biological function of these components, which are mainly present in neural membranes (and especially enriched in presynaptic membranes), remain obscure. They appear to play a role in many physiological functions, but the underlying molecular mechanisms still need to be elucidated. Next to their somewhat mysterious physiological functions, gangliosides are involved in the pathophysiology of several neurological disorders. They appear to be the initial targets of anti-ganglioside antibodies in autoimmune diseases like Guillain-Barré syndrome and Miller Fisher syndrome, and also play an important role as receptors for a number of neurotoxins. This thesis deals with the role of gangliosides and anti-ganglioside antibodies in both the physiology and the pathophysiology of the motor nerve terminal.

Gangliosides

Structure

The plasma membrane is an external, limiting bilayer between the cell and its environment, consisting mostly of lipids and proteins. There are three main classes of lipids in cell membranes: glycerol-lipids (e.g. phospholipids), sphingolipids, and sterols (e.g. cholesterol) (Breckenbridge et al., 1972). One subgroup of the (glyco) sphingolipids is formed by the gangliosides. These are amphipathic compounds composed of both sugar and lipid (ceramide) moieties, the latter of which consists of a fatty acid and a sphingoid base. Gangliosides have at least 3 sugar molecules, one of which must be a sialic acid: *N*-acetylneuraminic acid (NeuAc) or less commonly *N*-glycoloyl-neuraminic acid (NeuGc)*, or a NeuAc analogue in which the amine group is replaced by OH. The negatively charged sialic acid is the sugar that differentiates gangliosides from neutral glycosphingolipids and sulfatides. The structural formula of ganglioside GM2, as an example, is shown in Figure 1.1.

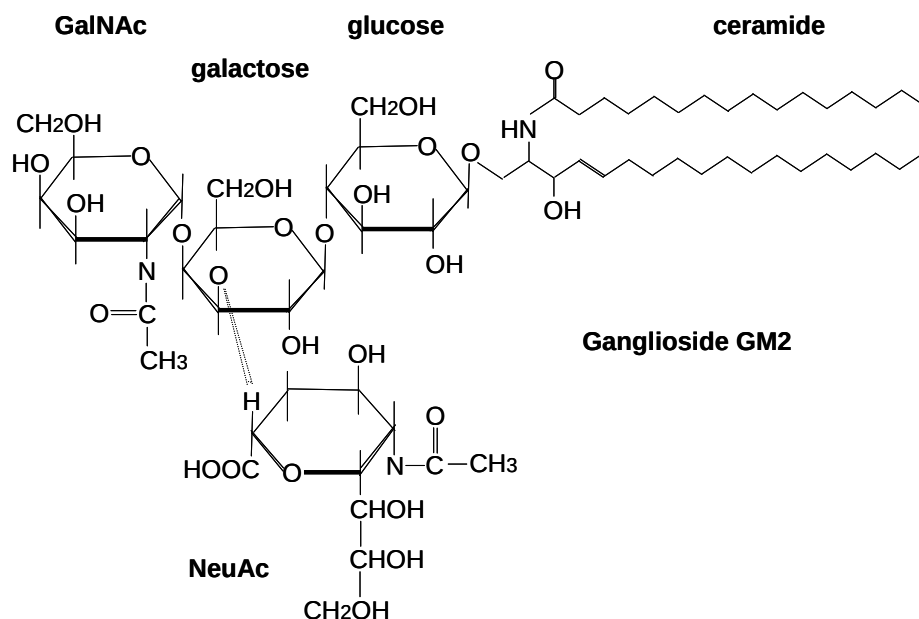


Figure 1.1. Structure of ganglioside GM2

Gangliosides are located primarily in the outer leaflet of plasma membranes with the hydrophobic ceramide inserted into the lipid bilayer and the hydrophilic headgroup consisting of neutral sugar molecules and sialic acids protruding into the extracellular space (Figure 1.2). In addition, a small proportion of the gangliosides is localized in the mitochondria and the endoplasmic reticulum inside the cell. Gangliosides are present in most cell types, but are especially abundant in nerve cells; they comprise up to 15% of the total amount of lipid in the vertebrate nervous system (d'Azzo et al., 2006) and are enriched in presynaptic membranes (Ledeen et al., 1993).

* Among mammals, the human species is very unique for the lack of NeuGc in any normal tissue. It is part of the only 1.23% genetic difference between human and chimpanzee (Suzuki, 2006).

Furthermore, gangliosides can also occur in a non-cell associated state in blood plasma and the cerebrospinal fluid and a constant exchange between free and membrane-bound gangliosides takes place (Bergelson, 1995).

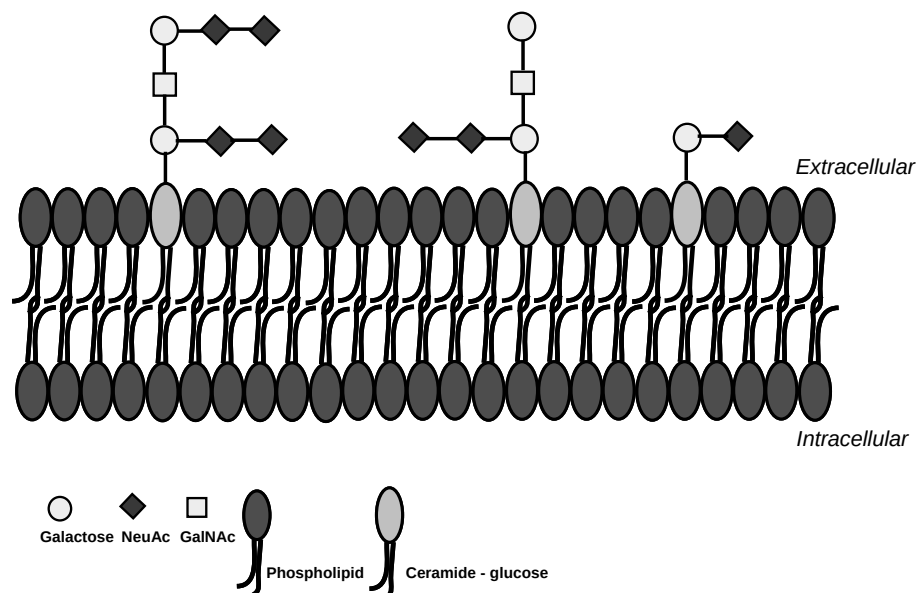


Figure 1.2. Schematic overview of gangliosides inserted in the phospholipid bilayer of the cell membrane. The hydrophilic portions of the gangliosides protrude into the extracellular space.

Biosynthesis

The biosynthesis of all sphingolipids starts in the endoplasmatic reticulum and continues in the Golgi apparatus. Ceramide is the precursor of the sphingolipids. A crucial (as well as rate limiting) step in biosynthesis of sphingolipids is its transport from the endoplasmatic reticulum to the Golgi apparatus with the aid of the transfer protein CERT (Kumagai et al., 2005; Morales et al., 2007). Glycosylation reactions* take place in the Golgi apparatus and after synthesis is completed, the gangliosides (as well as the other glycosphingolipids) reach their final destination in the cell membrane through vesicular exocytotic membrane flow (Kolter and Sandhoff, 2006). They are inserted in the outer leaflet of the plasma membrane.

The biosynthesis of the heterogenous family of gangliosides is a stepwise process starting with ceramide as core lipid (Figure 1.3). Through addition of acetylgalactosamine, galactose, and sialic acid moieties, the different gangliosides are formed. These additions are catalyzed by highly specific membrane-bound glycosyltransferases. The synthesis of lactosylceramide (LacCer) and the simplest gangliosides GM3, GD3 and GT3 occurs in a compartment more proximal in the Golgi apparatus than the one in which the synthesis of the complex gangliosides (e.g. GM2, GD2 and GD1b) is carried out (Maccioni, 2007; Giraudo and Maccioni, 2003).

* Glycosylation is the process of linking saccharides and is catalyzed by glycosyltransferases.

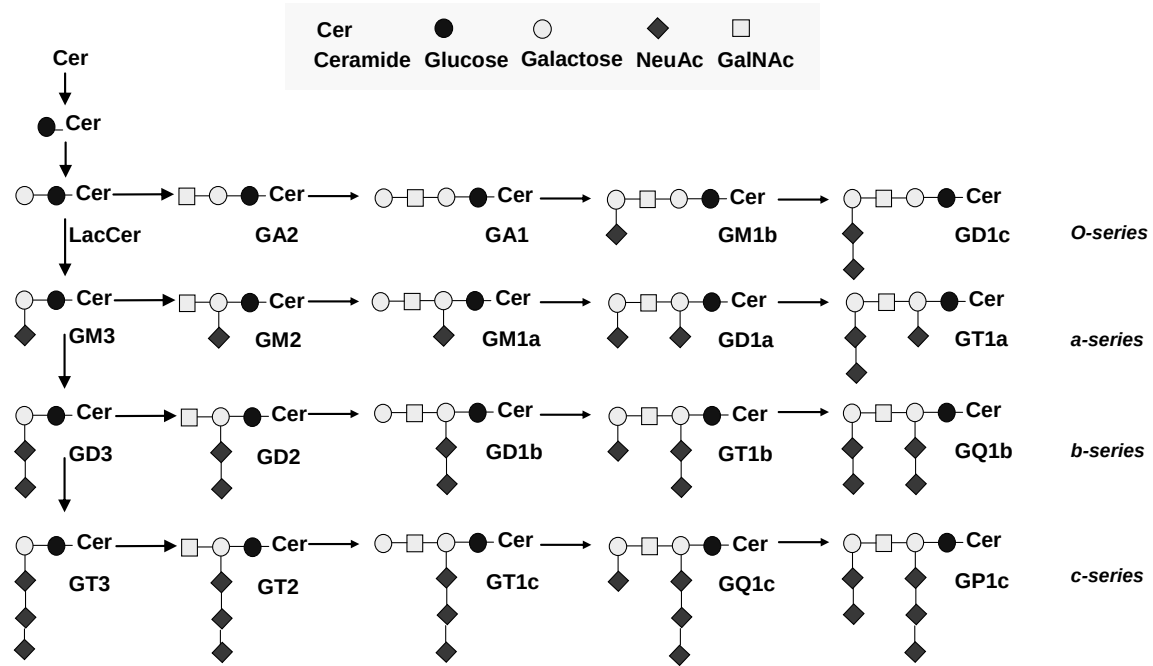


Figure 1.3. Simplified scheme of the ganglioside stepwise biosynthesis. Arrows point in the direction of the sequential step in the synthesis.

Besides anabolic processes, gangliosides also undergo catabolic processes involving a number of lysosomal hydrolases (Kolter and Sandhoff, 2005). Together, these activities are responsible for the expression of a single ganglioside species. Disruption of ganglioside metabolism is associated with several neurodegenerative disorders that can be lethal. Gangliosidoses (lysosomal storage disorders) are caused by impaired catabolism due to a genetic mutation in a degrading enzyme (Brunetti-Pierri and Scaglia, 2008; Paw et al., 1989; Kolter and Sandhoff, 1998; Kolter and Sandhoff, 2006; Buccoliero et al., 2004) and in Huntington's disease an imbalance in ganglioside metabolism is thought to be a pathological factor (Desplats et al., 2007). Infantile-onset symptomatic epilepsy syndrome is the first described disease in the anabolic pathway of gangliosides. It is caused by a loss-of-function mutation in GM3synthase (Simpson et al., 2004). Ganglioside compositions in the cell membranes can differ between animal species, even within the mammalian group (Saito and Sugiyama, 2001).

Function

In addition to their role as surface markers in the outer leaflet of cell membranes, gangliosides are thought to have multiple other biological functions. As gangliosides carry one or more negative charges in their headgroup, they can contribute significantly to the surface charge of the cell. Gangliosides can therefore modulate ion-interactions of proteins, in particular with protons and Ca^{2+} -ions, and can affect functioning of voltage-gated ion-channels in the membrane (Wang et al., 1999a; Wang et al., 1999b; Wang et al., 1996; Slomiany et al., 1992; Hayashi et al., 1984; Duan et al., 2006; Tanaka et al., 1997).

In general, gangliosides play a role in both inter- and intracellular signalling processes (Allende and Proia, 2002; Buccoliero and Futerman, 2003; Hashiramoto et al., 2006; Ravichandra and Joshi, 1999) and are thought to influence intracellular Ca^{2+}

homeostasis (Ledeen and Wu, 2006a; Ledeen and Wu, 2006b; Ginzburg et al., 2008). Wu and colleagues (2004) showed an impaired regulation of Ca^{2+} in cultured cerebellar granule neurons from mice that only express gangliosides GM3 and GD3. In the presence of a depolarizing concentration K^+ , these cells showed persistent elevation of intracellular Ca^{2+} leading to apoptosis and cell destruction. Gangliosides also play a role in the immune system (inhibition of cellular immune responses), temperature adaptation (Kappel et al., 2000), cell maintenance and repair, and apoptosis (Hakomori, 2002; d'Azzo et al., 2006; Malisan and Testi, 2002). It was for example found that GM3 act as a modulator of neuronal cell death, and that accumulation of GD3 can alter mitochondrial function and thereby induce cell death in a caspase-dependent manner (Sohn et al., 2006; De Maria et al., 1997; Morales et al., 2007).

More specialized roles for gangliosides are amyloid β protein binding (which is a key pathological hallmark in Alzheimer's disease (Lin et al., 2008; Peters et al., 2009)) and spermatogenesis and testosterone transport (Sandhoff et al., 2005). Furthermore, gangliosides can act as receptors for viruses, bacterial toxins (e.g. GM1 for cholera toxin and GQ1b for botulinum toxin) (Baldwin et al., 2007; Kozaki et al., 1998; Wu et al., 2007; Hansson et al., 1977; Fishman, 1982), and mammalian adhesion molecules, and have also been thought to be the autoimmune targets in immune-mediated neuropathies (e.g. the Guillain-Barré syndrome, see below). They also play a role in tumor growth (Choi et al., 2008; Hettmer et al., 2005), diabetes (especially the C-series gangliosides) (Saito et al., 1999; Gillard et al., 1989) and neurodegenerative diseases (Saito et al., 2007; Barrier et al., 2007, Lin et al., 2008).

Since gangliosides are so abundant in neuronal tissue, they are believed to be important factors in neuron-specific functions. For example it was found that external addition of GQ1b to the rat brain improved spatial learning and memory of the animal, possibly by modulation of NMDA receptors in hippocampal neurons (Jung et al., 2008). Ganglioside compositions change during life span (Svennerholm et al., 1997; Ngamukote et al., 2007), therefore gangliosides are thought to play a role in neural development, brain maturation and (at later age) maintenance and regeneration (Itoh et al., 2001; Wu et al., 2007; Desplats et al., 2007; Yamashita et al., 2005; Lehmann et al., 2007; Valaperta et al., 2007; Susuki et al., 2007b; Izumi et al., 1993). The gangliosides GM3 and GD3 were found to be relatively abundant in the early embryonic brain but decrease rapidly in later stages (Ngamukote et al., 2007). The function of this variable ganglioside expression during embryogenesis is quite elusive, as it was also found that mice with a cell-specific deletion for the *Ugcg* gene in the brain, thereby not expressing any gangliosides (and their precursors) in the brain, do not develop neural defects till after birth. These mice are born indistinguishable from their wildtype littermates, which at least suggests that gangliosides are not essential during embryonic ontogenesis of the nervous system (Jenneman et al., 2005). GD3 though, is thought to be a modulator of the ageing process, as its expression was found to increase again during the process of normal ageing (Malisan and Testi, 2002). And experimental results showed that ganglioside 9-O-acetyl GD3* is re-expressed during periods of regeneration after a sciatic nerve axon had been crushed (Ribeiro-Resende et al., 2007). Gangliosides are also known to be receptors for myelin-associated glycoprotein (MAG), an enhancer of axon-myelin stability, but also

* 9-O-Acetyl GD3 is a derivative of GD3 with an acetyl group attached to position 9 of the terminal sialic acid residue.

a potent inhibitor of axonal regeneration (after injury) (Yang et al., 1996; Cao et al., 2007; Vyas et al., 2002). Especially GT1b, GD1a and GQ1ba appear to be potent support molecules for MAG, while GD3 does not bind (Yang et al., 1996; Schnaar and Lopez, 2009).

In addition to age-dependent change of ganglioside compositions, differences in ganglioside expression can also be found at different sites in the body (Gong et al., 2002). For example, GQ1b gangliosides are especially abundant in the ocular nerves while GM1 is more expressed in the ventral than in the dorsal roots, and GT1a more in the lower than the upper cranial nerves (Hughes and Cornblath, 2005).

Furthermore gangliosides show subcellular concentration in lipid rafts in the membrane, also called microdomains. These are small regions enriched in cholesterol and sphingolipids that exhibit compartmentalized cellular processes (Asano et al., 2009; Sonnino et al., 2007; Crespo et al., 2004; Prinetti et al., 2009; Hakomori, 2002; Fujita et al., 2007). Within lipid rafts in the presynaptic membrane, important proteins of the neurotransmitter release machinery co-localize with gangliosides (Salaün et al., 2004; Lang et al., 2001; Taverna et al., 2004), which implies that gangliosides participate in neurotransmitter exocytosis. Several studies have shown that application of exogenous gangliosides indeed influences synaptic transmission; e.g. in brain slice preparations the addition of GM1 or GQ1b was found to enhance nerve potentiation (Ramirez et al., 1990; Egorushkina et al., 1993; Wieraszko and Seifert, 1985; Furuse et al., 1998) and a change in ganglioside pattern through reduction of bound sialic acids, resulted in a decrease of cholinergic transmission efficiency, i.e. abolishment of evoked potentials (Wieraszko and Seifert, 1984). Tanaka and colleagues (1997) showed that bath-applied GM1 and GQ1b increase K^+ -evoked neurotransmitter release from mouse brain synaptosomes, presumably through modulation of N-type Ca^{2+} channels. Ca^{2+} -ions are important players in the process of neurotransmitter release and in addition to Ca^{2+} channel modulation gangliosides are also able to directly bind these ions (Rahmann et al., 1992). These *in vitro* results suggest a modulating role in neurotransmitter release. The synthesis and levels of the neurotransmitter itself however, were not found to be affected by exogenous application of gangliosides (Tanaka et al., 1997).

Neuromuscular junction

Structure and function

Motor neurons in the brain stem and the spinal cord give rise to axons that branch intramuscularly to provide peripheral single nerve terminals at each muscle fibre. The motor neuron and the muscle fibres it innervates together form a motor unit (Figure 1.4). A muscle fibre can be innervated by one motor neuron only, except for the extraocular muscle fibres, which can be innervated by axons from multiple motor neurons. The neuromuscular junction (NMJ) is a specialized chemical synapse where transmission of information takes place between the axon of a motor neuron and a skeletal muscle fibre. A very short distance separating the motor nerve from the striated muscle characterizes the place where transmission of the impulse from nerve to muscle can occur. The presynaptic and postsynaptic membranes remain separated though by a basal lamina at every point and the nerve terminal branches are covered

by the non-myelinating terminal Schwann cells (Sanes and Lichtman, 1999). The synaptic cleft is about 60 nm wide.

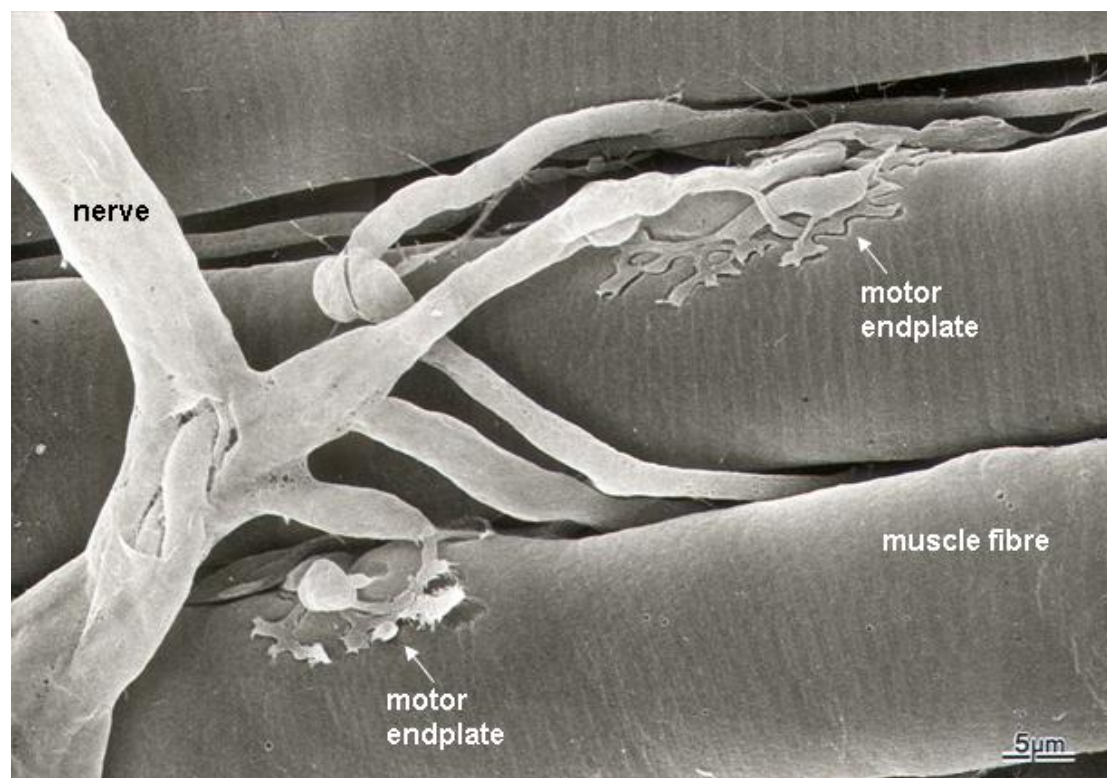


Figure 1.4. Scanning electron micrograph of two NMJs in the sternothyroid muscle of the Chinese hamster
The intramuscular nerve produces side branches, which terminate in motor end-plates on the muscle nerve surface. Picture is used with approval of Dr. Desaki.

The NMJ contains many highly specialized sites, both pre- and postsynaptic, that are differentiated for organisation and mediation of neurotransmission. In vertebrate NMJs the neurotransmitter is acetylcholine (ACh), which is synthesized in the cytosol of the nerve endings from acetyl coenzyme A and choline by the enzyme choline acetyltransferase. A single nerve terminal has approximately 200,000 synaptic vesicles, each containing ACh. Under controlled conditions fusion of these vesicles with the presynaptic membrane results in transmitter release into the synaptic cleft. The released transmitter diffuses in about 0.5 ms across the extracellular synaptic space and binds to nicotinic ACh receptors (nAChRs) on the postsynaptic cell. After activation of the nAChRs the neurotransmitter is catabolized by acetylcholinesterase in the extracellular synaptic space.

The arrangement of synaptic vesicles in the nerve terminal constitutes at least two different pools: a readily releasable pool (RRP) near the active zones and storage pools more upstream in the nerve terminal. At rest, the RRP contains about 20% of the vesicles present in the nerve terminal (Richards et al., 2003; Petrov et al., 2008). Prior to exocytosis a process called docking takes place, when the synaptic vesicles move into active zones extremely close to the membrane. This is followed by priming: the formation of the so-called SNARE complex, which includes synaptobrevin at the vesicle membrane, and SNAP25 and syntaxin at the cell membrane. Also located in the active zones are the voltage-gated Ca^{2+} channels (the

principal Ca^{2+} channel in the mammalian NMJs is the P/Q-type or $\text{Ca}_v2.1$ -type channel (Urbano et al., 2008)), mediating calcium influx in response to a presynaptic impulse. By binding to synaptotagmin, which is a synaptic vesicle surface protein, calcium stimulates the formation of a fusion pore between a vesicle and the presynaptic membrane, leading to the release of neurotransmitter into the synaptic cleft (Hua et al., 2007; Figure 1.5). After the fusion event has taken place, new synaptic vesicles are formed by endocytosis and they are refilled with neurotransmitter in the nerve terminal. This constant turnover of vesicles requires energy which is provided by the numerous mitochondria present in the nerve terminal (Lisman et al., 2007).

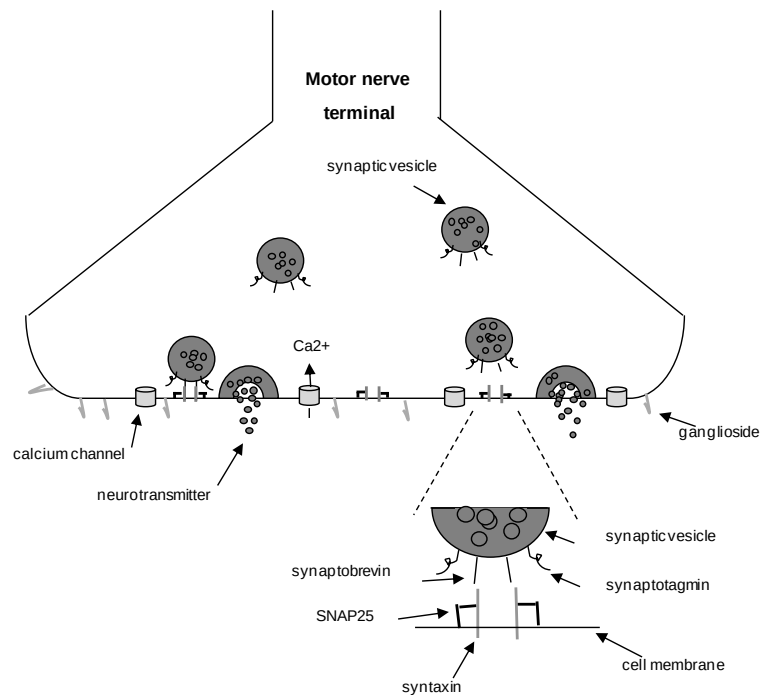


Figure 1.5. Neurotransmitter release at the motor nerve terminal

The postsynaptic membrane is specialized to respond adequately to the released neurotransmitter. It contains a high concentration of nAChRs, which are ligand-gated ion channels. They predominantly accumulate at the endplates where lipid rafts play an important role in regulating AChR clustering. The clustering is, amongst others, agrin, muscle-specific kinase, and rapsyn dependent (Zhu et al., 2006; Lin et al., 2001; Chen et al., 2007b). The AChR is a Na^+/K^+ channel. When activated through binding of ACh it opens and allows for a relative large influx of Na^+ -ions and a smaller flow outwards of K^+ -ions, the net influx of positivity leading to a depolarization of the muscle membrane. These changes in electric properties of the membrane are termed "end plate potential" (EPP). If the EPP is sufficiently large, a muscle fibre action potential is triggered by opening of the voltage-gated Na^+ channels, leading to contraction of the muscle. The total charge of an EPP required to produce an action potential, has to overcome a threshold value. During normal activity the released amount of neurotransmitter is abundantly greater than the required threshold, which allows a safety margin for more stressful situations and ensures the NMJ to be a very reliable synapse (Wood and Slater, 1997; Wood and Slater, 2001).

Electrophysiological NMJ Analysis

The nAChR opening-induced changes in postsynaptic muscle cell membrane potential can be measured *in vitro* with intracellular recordings using a microelectrode (Figure 1.6). A miniature endplate potential (MEPP) represents the release of a single vesicle of neurotransmitter from the presynaptic nerve terminal into the synaptic cleft, resulting in the depolarization of the postsynaptic muscle membrane by about 1 mV. MEPPs appear spontaneously with a frequency of ~1 per second in the mouse diaphragm muscle. The amount of neurotransmitter in a vesicle constitutes a basic unit, called a quantum (about 6,000 to 10,000 molecules of ACh). A second type of depolarization is the endplate potential (EPP), which represents the release of ACh of several vesicles upon stimulation of the nerve, and is actually a summation of the released MEPPs that would have been induced by the individual quanta. Features of both MEPP and EPP can vary, depending on several factors like species, muscle type and age. Example traces are shown in Figure 1.6. The amplitudes of EPPs and MEPPs can be used to calculate the amount of quanta that is released upon a single stimulation of the nerve, the quantal content (QC). To calculate the QC at each NMJ the following formulas are used:

First the amplitudes are adjusted to a standard resting potential of -75 mV:

$$(M)EPP_n = (M)EPP_m * ((V_{std} - V_{ACh}) / V_m)$$

(M)EPP_n: normalized amplitude

(M)EPP_m: the amplitude as measured

V_{std}: the standard resting membrane potential (-75 mV by choice)

V_{ACh}: the reversal potential for the ACh induced current, which is 0 mV at the NMJ, according to Magleby and Stevens (1972).

V_m: the resting membrane potential as measured

The McLachlan and Martin (1981) equation is then used to correct for the non-linear summation (i.e. the non-linear relationship between endplate current and potential as a result of a reduced driving force when additional depolarization is added due to AChR openings by release of additional ACh quanta):

$$EPP_c = EPP_n / (1 - (0.8 * EPP_n / V_{std}))$$

EPP_c: calculated EPP amplitude

EPP_n: normalized EPP amplitude

0.8 is a factor dependent on the duration of transmitter action relative to the membrane time constant (McLachlan and Martin, 1980). This number accounts for mice muscle only (e.g. for frogs it's 0.55).

V_{std}: 75mV is again chosen as the standard resting membrane potential

Finally the QC can be calculated as: $QC = EPP_c / MEPP_n$

The EPP would normally cause an action potential, causing the muscle fibre to contract. A selective skeletal muscle sodium channel blocker, μ -conotoxin GIIIB, can be used to eliminate muscle action potentials, which allows microelectrode measurement inside the muscle fibre. This toxin is a peptide isolated from the venom of the marine cone snail *Conus Geographus*.

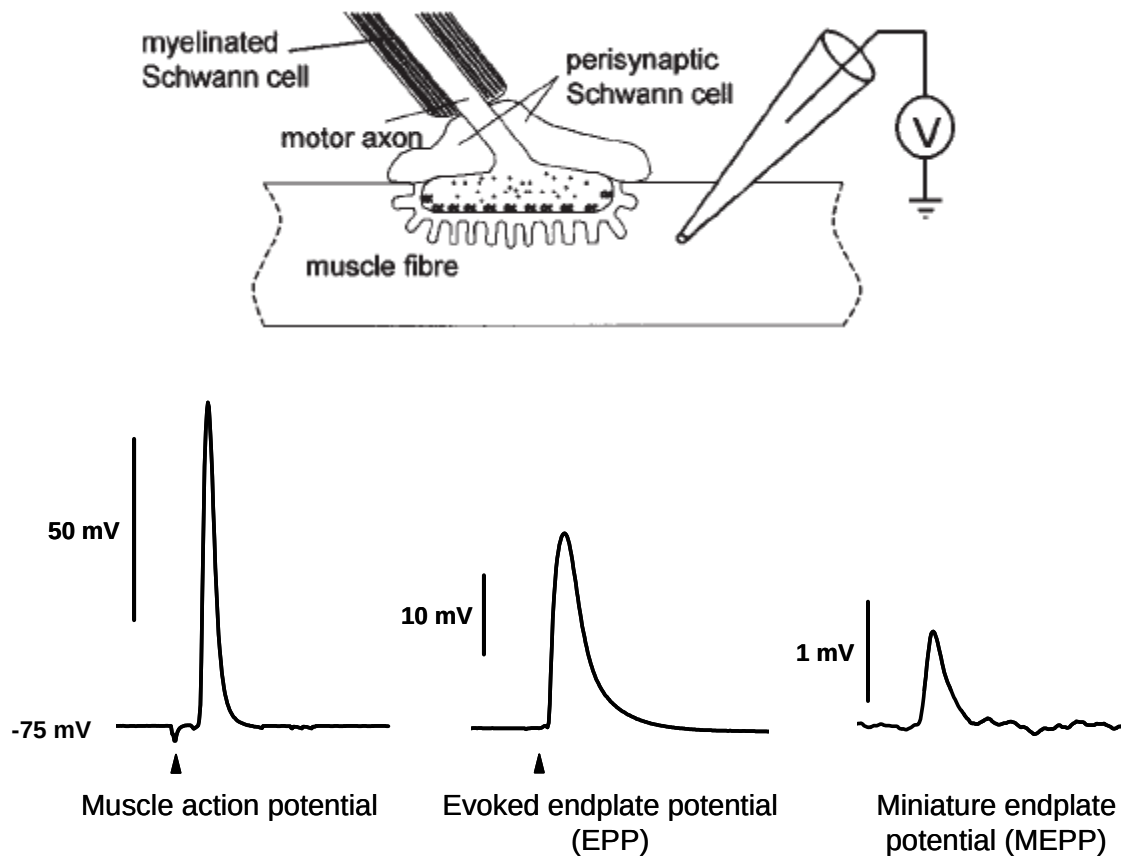


Figure 1.6. Measurement at the NMJ

Top scheme of motor endplate is adapted from O'Hanlon et al. (2002) and shows the micro-electrode (on the right) inserted into the muscle fibre. This way, alterations in the membrane potential of the muscle fibre can be recorded. These alterations are the result of ACh molecules that have been released from the motor nerve terminal (spontaneously or evoked) and bound to postsynaptic receptors. Below, examples traces of the three signals that can be measured at the NMJ are shown: The membrane potential baseline is -75 mV. Successful transmission results in a muscle action potential. When a selective muscle sodium channel blocker is applied (μ -conotoxin GIIIB), the action potential is reduced to an EPP. A MEPP is the result of the spontaneous release of a quantum ACh from a single vesicle of ACh.

▲ Indicate moment of stimulation

The dissected mouse diaphragm muscle with phrenic nerve attached is a most suitable preparation for muscle-nerve synapse analyses. It is a flat muscle that can be pinned out on a silicon rubber-lined base of a petri dish. It is only 10 fibres thick which makes it very accessible for the $\sim 1 \mu\text{m}$ tip of the glass microelectrode. Furthermore the phrenic nerve is very well defined in the NMJ regions, which makes it easy to find the points of interest, and the nerve can be dissected over more than a centimetre, which is long enough to place it over a bipolar stimulation electrode.

In addition to intracellular measurements it is also possible to visually assess the muscle nerve preparation and score twitching of the individual muscle fibres when the preparation is challenged with toxins or antibodies (as was described by Jacobs et al., 2002).

Ganglioside-mediated NMJ pathophysiology

Toxins

The NMJ, unlike most parts of the nervous system, is accessible to factors circulating in the blood. This can be a disadvantage when for example botulinum neurotoxin enters the body. This toxin is produced by *Clostridia* bacteria and can disable the exocytotic machinery at the motor nerve terminal through cleavage of one of the molecules involved in the SNARE-complex (each serotype of the toxin cleaves a specific protein). The cleavage results in blockage of the release of ACh from the motor nerve terminal and leads to paralysis of the muscle (Dolly and Aoki, 2006; Brunger and Rummel, 2009). It was already shown in the 1980's that *Clostridium botulinum* neurotoxin interacts with gangliosides (Kitamura et al., 1980; Hayes, 1979) and later research indicated that this toxin enters the motor nerve terminal through receptor mediated endocytosis with gangliosides in the role of the receptor (Bullens et al., 2002; Yowler and Schengrund, 2004; Verderio et al., 2006). Also for tetanus toxin it is since long recognized that gangliosides act as a receptor (Halpern and Loftus, 1993). This other member of the *Clostridial* neurotoxin family enters the NMJ and then travels to the spinal cord by retrograde axonal transport. Tetanus blocks the release of glycine or γ -aminobutyric acid from inhibitory neurons, which results in spastic paralysis (Middlebrook and Dorland, 1984; Brunger and Rummel, 2009).

Another neurotoxin that has the NMJ as target site is α -latrotoxin (α LTx). α LTx is the active component of the Black Widow spider venom and can form non-selective pores within the nerve membrane. These pores allow free passage of ions and small molecules. Due to an increasing concentration of calcium inside the cell, massive uncontrolled exocytosis of neurotransmitter takes place. This massive quantal release of ACh can be measured as an enormous increase in MEPP frequency and is visible by twitching of the affected muscle fibres. As with the *Clostridia* bacteria toxins, α LTx finally leads to muscle paralysis (Hu et al., 2006; Peterson, 2006).

Anti-ganglioside antibodies

As described above, gangliosides are abundant in neuronal tissue and they are especially enriched at NMJs. In *in vitro* studies using mouse muscle-nerve preparations it was shown that murine monoclonal anti-ganglioside antibodies were able to bind presynaptic gangliosides at the NMJ (Paparounas et al., 1999; Plomp et al., 1999). It was reported that the events following the antibody-binding closely resembled the effects of α LTx, as an increase in MEPP frequency was observed. Also serum from patients with Miller Fisher syndrome (see below), which contains high titres of anti-GQ1b antibodies, was found to induce an increase in spontaneous ACh release when added to an *ex vivo* mouse diaphragm (Bullens et al., 2000; Jacobs et al., 2002). As with the neurotoxin, the final result was a blockade of transmission and muscle paralysis, but in contrast to α LTx the antibodies depended on the activation of the complement cascade to achieve this. Complement depositions were found at NMJs of the mouse tissue preparations used in these incubation studies (Jacobs et al., 2002; Goodyear et al., 1999; Plomp et al., 1999; Halstead et al., 2004; Goodfellow et al., 2005). And this complement-dependency was further confirmed in an *ex vivo* assay in the mouse NMJ, with complement component C5-deficient serum: the exogenous applied anti-ganglioside antibodies were not able to induce the α LTx-like effects (Plomp et al., 1999). Component C5 is, when spliced into C5a and C5b, the

first component of the membrane attack complex (MAC; C5b-C9), which is a pore forming molecule.

Based on experimental studies in mice, the following neuropathophysiological mechanism has been suggested: the anti-ganglioside antibodies bind gangliosides in the presynaptic membrane, leading to induction of the innate immune response. The activated complement system culminates into the formation of MAC. This final component of the complement system is able to cause lysis of the nerve cell through the formation of pores in the membrane. Ca^{2+} -ions can then freely move into the cell and trigger the release mechanism of ACh in an unregulated way. This finally results in an exhaustion of neurotransmitter in the distal end of the nerve and leads to impairment of communication between nerve and muscle (i.e. transmission block), which causes paralysis of the muscle (Rinaldi and Willison, 2008; Willison and Plomp, 2008). This pathological process and the involvement of complement in anti-ganglioside antibody-mediated NMJ pathophysiology is extensively reviewed and discussed in chapter 3.1.

In addition to complement-activation, other factors of the immune system can be activated by bound anti-ganglioside antibodies, e.g. T cells (Csurhes et al., 2005), or more in general leukocytes, can be recruited. This can lead to lymphocyte and macrophage infiltrations in and around the NMJ and contributes to inflammation of the nerve cell (Van Sorge et al., 2007a; Van Sorge et al., 2007b). On top of the destructing effects of anti-ganglioside antibodies to the nerve terminal, it is also shown that these antibodies can play an inhibiting role in axonal regeneration (Lehmann et al., 2007). This could indicate a limitation for recovery of injured axons. Several studies have claimed that anti-ganglioside antibodies were also able to inhibit voltage-dependent Ca^{2+} -channels, as was observed in rat Purkinje cells (Nakatani et al., 2009), PC12 cells (Nakatani et al., 2007a; Nakatani et al., 2007b), and at mouse diaphragms NMJs (Buchwald et al., 2007; Ortiz et al., 2001). The effects were reversible, and it was therefore hypothesized that muscle weakness induced by anti-ganglioside antibodies can be independent of complement-involvement and might be directly caused by a dysfunction of Ca^{2+} -channels in the NMJ. This impaired impulse conduction leads to little structural change and has potential for rapid recovery. Another pathophysiological action of anti-ganglioside antibodies was found after a two week continued passive transfer of anti-GM2 IgM to mouse NMJs. Again no complement deposition could be observed, and no NMJ lesions or block of neurotransmission were encountered. But morphology showed nerve terminal growth and retraction changes, and with electrophysiology, a significant reduction in evoked neurotransmitter release was found (Santafé et al., 2005). These observations suggest that in addition to the complement-mediated nerve damage induced by anti-ganglioside antibodies, other complement-independent actions of anti-ganglioside antibodies can also play a role in nerve-terminal pathophysiology (Santafé et al., 2008).

Furthermore, though outside the scope of this thesis, other molecules than gangliosides at the NMJ are also known to act as target sites in antibody-mediated autoimmune diseases. Examples are the postsynaptic AChRs (or muscle-specific tyrosine kinases) in myasthenia gravis (Gilhus, 2009), presynaptic voltage-gated Ca^{2+} -channels in Lambert-Eaton myasthenic syndrome (Motomura et al., 1997; Lennon et al., 1995), and presynaptic voltage-gated K^{+} -channels in Isaac's syndrome (Arimura et al., 1997).

Guillain-Barré syndrome and Miller Fisher syndrome

Clinical symptoms

In 1916 three French neurologists, Georges Guillain, Jean-Alexandre Barré, and André Strohl, reported two cases of acute areflexic paralysis in soldiers that spontaneously recovered. In addition to the clinical symptoms, laboratory findings showed increased protein concentration in the cerebrospinal fluid with a normal white blood cell count (Guillain et al., 1916). Although several clinical cases were already described in the 19th century (Brody et al., 1994) and although it was Strohl who performed the electrophysiological tests on the soldiers, the combination of features became known as the Guillain-Barré syndrome (GBS) (Pritchard and Hughes, 2004).

GBS is a post-infectious peripheral neuropathy that causes acute neuromuscular failure. GBS is recognized as a group of conditions with diverse pathology and pathogenesis, but all types present with (sub)acute neuropathy, defined as a progressive onset of limb weakness. This is self-limited and reaches its nadir within two to four weeks. In severe cases, paralysis involves the nerves feeding the brainstem, and patients may lose the ability to swallow or even breathe. Respiratory failure is the most severe complication of GBS, and mechanical ventilation is required in 20 to 30% of the patients (Durand et al., 2006).

Although it is a disease with a self-limiting nature, it takes weeks to months to recover (partially) and there still is a considerable mortality (5-10%) from this disorder. The overall worldwide incidence of GBS is estimated at 1.1 to 1.8 per 100,000/year, with lower rates in children and an increase in incidence after the age of 50 (McGrogan et al., 2008). For The Netherlands this comes down to about 200 people per year that are diagnosed with GBS. Slightly more males are affected than females, which is unusual for an autoimmune disease.

GBS has highly variable clinical forms, of which the incidences are not equally distributed geographically (Shafqat et al., 2006; Cheng et al., 2006; Willison and Yuki, 2002). There are at least five subtypes:

- Acute inflammatory demyelinating polyradiculoneuropathy (AIDP) is the most common form of GBS in Western countries. The site of action appears to be the myelin sheath and the Schwann cells of sensory and motor nerves (Hafer-Macko et al., 1996a).
- Acute motor axonal neuropathy (AMAN) is a pure motor form of GBS and occurs with higher frequency in elderly people (Leung, 2008). The site of action is the nerve axolemmal membrane (Hafer-Macko et al., 1996b).
- Acute motor-sensory axonal neuropathy (AMSAN) is characterized by early axonal degeneration of both motor and sensory fibres. AMAN and AMSAN were first described in 1986 as one axonal variant of GBS by Feasby and colleagues (1986).
- Recurrent GBS or chronic inflammatory demyelinating polyradiculoneuropathy (CIDP) is a chronic condition, distinguished from GBS by its temporal pattern and its potential for clinical relapse (Csurhes et al., 2005).
- Miller Fisher syndrome (MFS) is a related condition, commonly thought to be a variant of GBS. In contrast to the generalized and often severe limb, respiratory and axial weakness, and sensory loss that occur in the other subtypes of GBS, the manifestations of MFS are restricted to limb ataxia, tendon reflex loss, and extraocular muscle paralysis. MFS accounts for 5-10% of the GBS cases and has a good prognosis (Flachenecker, 2006).

It is unclear whether Bickerstaff's brainstem encephalitis (BBE) is a distinct disease or a subtype of MFS (Paparounas, 2004). BBE is characterized by ataxia, ophthalmoplegia, and disturbance of consciousness. Ito and colleagues (2008) postulate that BBE and MFS are not distinct from each other clinically, anatomically, or aetiologically, and therefore these two conditions represent the same autoimmune disease with variable involvement of the peripheral nervous system (PNS) and the central nervous system (CNS). Because of clinical and aetiological overlap, there are more conditions considered as subtypes of GBS, e.g. pharyngeal-cervical-brachial weakness and acute ophthalmoparesis (Willison and Yuki, 2002; Nagashima et al., 2007; Tatsumoto et al., 2006; Manganelli et al., 2009; Notturmo et al., 2008; Mori et al., 2001).

GBS typically has a three phase course: the progressive phase delineates the onset of symptoms (pain, numbness, paraesthesia and/or weakness in the limbs) leading to a peak-illness within 2-4 weeks. This is followed by a variable steady plateau phase, after which the final phase of recovery starts (Hughes and Cornblath, 2005). In a study from Forsberg and colleagues (2008) the experiences of patients during the initial phase of GBS were examined. The onset of the disease is characterized by a frightening rapid paralysis and deterioration of the body. Although the onset can be fast, the recovery tends to take several weeks to months, which means an extensive time of hospitalization, often followed by a further rehabilitation program. Patients therefore suffer severely, not only physically, but also mentally. In addition, it is reported that the long-term impact of GBS appears to reduce the quality of life, with persistent fatigue as one of the most disabling symptoms (Garssen et al., 2007; Rudolph et al., 2008; Rekan et al., 2009).

Anti-ganglioside antibodies

In about half of the GBS patients, elevated titres of anti-ganglioside antibodies are found in the acute phase serum and in MFS patients antibodies are found in even more than 90% of the cases (Lange et al., 2006; Willison et al., 1993; Van Doorn et al., 2008; Hafer-Macko et al., 1996b). The antibodies found in MFS patients are mostly anti-GQ1b antibodies. For the other clinical variants of GBS it is not possible to match a certain antibody to a certain subtype, although there are some combinations that occur more frequently. AMAN for example is associated with anti-GD1a and anti-GM1 IgG antibodies (Lopez et al., 2008; Yuki, 2007a; Matà et al., 2006). Kaida and colleagues (2004b) found "antibody-negative" GBS sera that did react with the gangliosides GD1a and GD1b in a complex form, but not with either ganglioside alone. Gangliosides along with other components as cholesterol are known to form lipid rafts in cell membranes, in which the carbohydrate portions of two different gangliosides may form a new conformational epitope. Kaida also found that the presence of antibodies against GD1a/GD1b and/or GD1b/GT1b complexes was (on the short term) significantly associated with a severe course of the disease and a requirement for mechanical ventilation (Kaida et al., 2007).

Clinical findings suggest that the concentration of particular gangliosides in the targeted membrane might determine the vulnerability to specific antibodies, as is clearly seen in MFS where predominantly the oculomotor nerves, which are rich in GQ1b gangliosides, are the most severely affected (Chiba et al., 1997; Chiba et al., 1993). A related mechanism seems present in genetically manipulated mice that lack part of the ganglioside family, but over-express the remaining ones. They show increasing vulnerability for anti-ganglioside antibodies, with binding specificity to these remaining gangliosides (Goodfellow et al., 2005; Bullens et al., 2002).

The anti-ganglioside antibodies are able to induce experimental pathogenic effects, as was described in the previous section, and therefore are likely to cause the clinical symptoms seen in GBS. It should be pointed out however that these antibodies are not found in all patients, so their pathogenic role remains somewhat speculative and it cannot completely be excluded that the presence of antibodies is a secondary phenomenon. On the other hand, also the presence of anti-ganglioside antibodies does not necessarily result in the development of GBS. This was found in rabbits that were passively immunized with anti-GM1 antibodies and only a subset of the animals developed AMAN (Van Sorge et al., 2007a). Also, some healthy human subjects may have anti-ganglioside antibodies in their plasma (Prendergast et al., 2004).

Molecular mimicry

About two third of the GBS patients, report an antecedent infectious disease, most of which are respiratory tract and gastrointestinal infections (Cheng et al., 2002; Yuki, 2001). A preceding infection with *Campylobacter jejuni* or cytomegalovirus (CMV) can be linked to GBS and therefore molecular mimicry and a cross-reactive immune response are thought to play a crucial part in the pathogenesis of the disease (Yuki, 1997; Taboada et al., 2007; Yuki, 2007a; Yuki, 2007b). Molecular mimicry refers to a structural resemblance of host-tissue and pathogens, leading to a dual recognition by a T- or B-cell receptor, and is therefore one of the proposed initiating factors in GBS (Bach, 2005; Albert and Inman, 1999). The pathogens that are found in GBS patients are mostly *C. jejuni* (associated with anti-GM1 and (cross-reactive) GQ1b antibodies) and CMV (associated with anti-GM2 antibodies) (Winer et al., 1988; Furiya et al., 2008). Furthermore, *Mycoplasma pneumoniae*, *Haemophilus influenzae* and Epstein-Barr virus can be the culprit and there are also case studies that describe a possible association between *Legionella pneumophila* or *Rickettsia prowazekii* (causing typhus) and GBS (Akyildiz et al., 2008; Lee et al., 2008; Yu et al., 2006). For *C. jejuni* it has been established that it has lipopolysaccharides* (LPS) epitopes that mimic certain gangliosides (Winer, 2008; Godschalk et al., 2007; Figure 1.7). Jacobs and colleagues (1996) showed an association with anti-GM1 antibodies in GBS patients and a preceding *C. jejuni* infection. Other studies (Moran et al., 2005; Ang et al., 2000) showed that the sera of rabbits that where immunized with GM1-mimicking *C. jejuni* lipooligosaccharides† (LOS) contained high titres of anti-LOS antibodies that where able to bind gangliosides in the peripheral nerve. Similar results were obtained in mice immunized with GT1a/GD3-like *C. jejuni* LPS (Goodyear et al., 1999). Perera and colleagues (2007) demonstrated in mice that only the gangliosides mimicking molecules of *C. jejuni*, and no other microbial cell components were required for the production of anti-ganglioside antibodies. It was also shown that the sialic acid residues of the *C. jejuni* LOS are essential for the ganglioside mimicry induction of GBS. A wildtype *C. jejuni* strain lost its pathological activity (i.e. triggering GBS) after a mutation that erased this crucial epitope (Xiang et al., 2006).

However, careful judgement is required because not all strains of the same pathogenic microbe can be linked to GBS (Godschalk et al., 2004) and the presence of a

* Lipopolysaccharides (LPS) are major components of most gram-negative bacterial outer membranes. They are composed of three regions: a lipid A component, a core of oligosaccharides, and an O-chain of repeating oligosaccharide units.

† Lipooligosaccharides (LOS) are LPSs that lack the O-chain

molecular mimicry site, is not necessarily related to the development of GBS as shown for *Campylobacter coli* (Funakoshi et al., 2006).

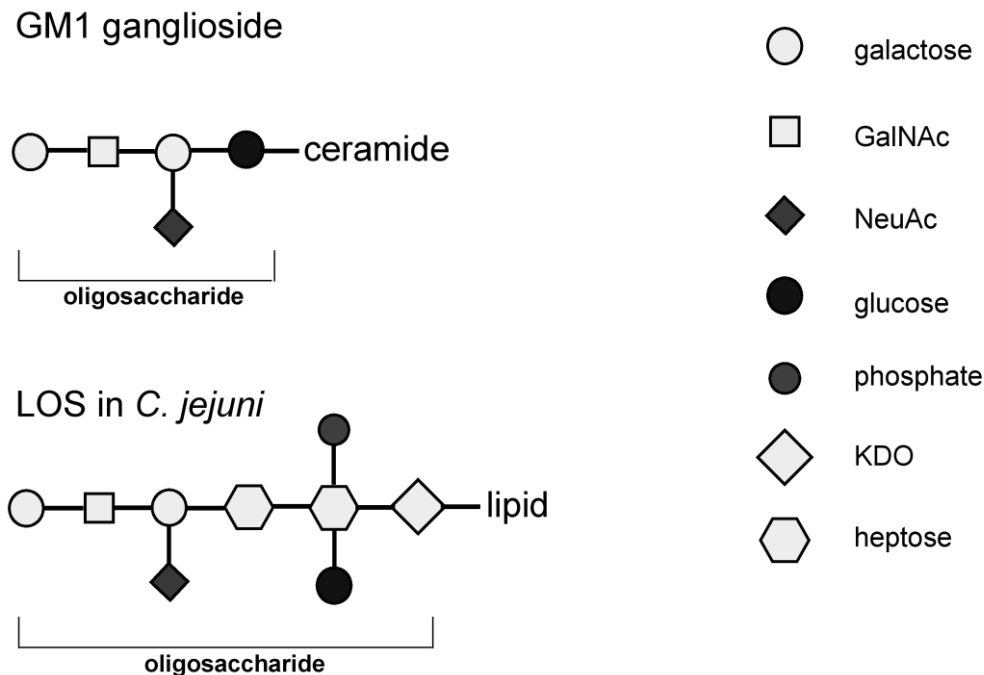


Figure 1.7. Picture of identical structure GM1 and the outer core of *C. jejuni* LOS
KDO: 3-deoxy-D-manno-2-octulosonic acid (picture is based on: Moran and Prendergast, 2001)

A study by Nachamkin and colleagues (2008) showed the presence of components in influenza vaccines that can induce anti-GM1 antibodies in mice. Several studies have examined the risk of a GBS outbreak after immunization programs (Haber et al., 2004; Lasky et al., 1998). However, no epidemiological evidence has been found for an increased vaccine-associated risk of GBS.

Complement involvement

The pathophysiological effects of anti-ganglioside antibodies are most likely to be complement-mediated, although some experimental results suggest complement-independent effects as well (see previous section). In nerve biopsies of GBS patients complement depositions have been found (Hafer-Macko et al., 1996a; Hafer-Macko et al., 1996b) and it was reported that elevated levels of anti-GM1 ganglioside antibodies by themselves do not cause nerve damage (Ilyas and Chen, 2007). *In vitro* studies in mice muscle-nerve preparations showed that complement was needed to obtain a pathologic effect of the anti-ganglioside antibodies at the NMJ (Jacobs et al., 2002; Goodyear et al., 1999; Plomp et al., 1999; Halstead et al., 2004; Goodfellow et al., 2005).

The complement system is part of the innate immune response against pathogens (Walport, 2001; Kawano, 2000). The activation of complement encompasses a cascade of enzymatically stimulated actions which involve splicing and binding of components (Figure 1.8). There are three pathways in the complement system, which all culminate into the formation of component C5, which is (when cleaved into C5a and C5b) the first component of the membrane attack complex (MAC; C5b-C9).

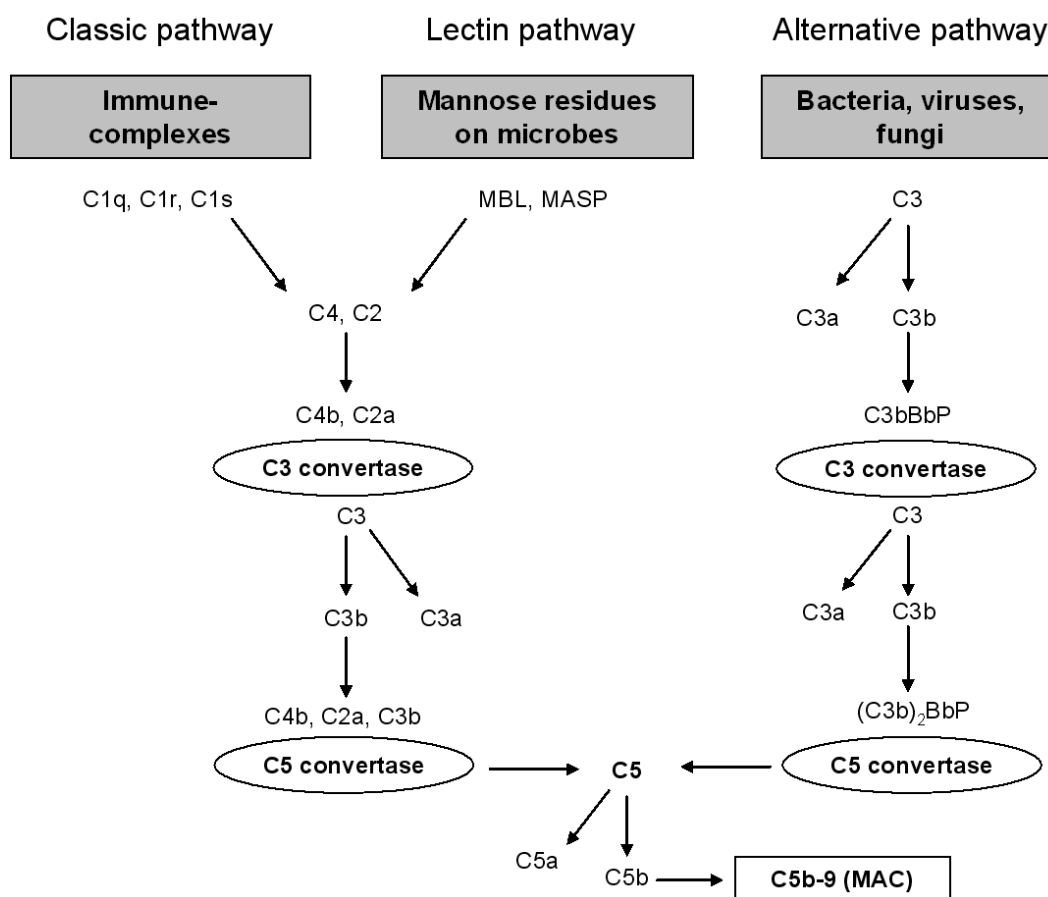


Figure 1.8. Scheme of complement cascade

The classical pathway is mainly activated by antibody-antigen binding. The lectin pathway starts with the binding of *mannose-binding lectin* (MBL) to carbohydrate surfaces and subsequent binding of *MBL-associated serine proteases* (MASPs). The alternative pathway is triggered by bacterial or viral surface components (for review see Walport, 2001).

Each component of the cascade has an inflammatory or cytotoxic function. For example the anaphylactic fragments C3a and C5a recruit and activate phagocytes and C4b and C3b label targets for phagocyte uptake by binding to them. MAC is able to cause cell lysis by disrupting cell membrane integrity.

It was found in mice muscle-nerve preparations that application of complement component C6-deficient serum following incubation with anti-ganglioside antibodies did not lead to neuropathophysiological damage. Although the complement system was initially triggered by the antibodies, the inability to create MAC protected the integrity of the membrane (Halstead et al., 2004). This further suggests that GBS is complement-mediated and that MAC is a necessary component for the proposed pathophysiological effects in this disease. Besides in GBS, the complement system is important in a number of other autoimmune and neurodegenerative diseases. Therefore, it is a very interesting target for therapeutics. Naturally occurring inhibitors are already present in the bloodstream and on cell membranes (the complement system is potentially very harmful and therefore it is extremely well-regulated) (Van Beek et al., 2005). Recently, many compounds have been discovered or developed for use as pharmacological complement inhibitor (Ricklin and Lambris, 2007). Chapters 3.2 and 3.3 describe two studies in which new complement inhibitors were assessed

for their ability to block the neuropathophysiological effects of anti-ganglioside antibodies at the mouse NMJ.

Genetic predisposition

The reason why a certain pathogen can induce the development of an autoimmune disease still needs to be elucidated. For example, the number of infections with *C. jejuni* in The Netherlands is estimated at 100,000 per year (Havelaar, 2002), while less than 200 persons per year are diagnosed with GBS. This raises the question about a possible (genetic) predisposition of the host, or a specific feature of the *C. jejuni* strain. An example of the latter could be that *cst-II* polymorphism in *C. jejuni* is closely related to autoantibody reactivity as well as to the neurological presentation of GBS (Koga et al., 2005; Godschalk et al., 2007; Yuki, 2007b). And polymorphisms in host CD1E and CD1A genes* were found to be associated with susceptibility to GBS in one study (Caporale et al., 2006), though this could not be confirmed in a larger study (Kuijf et al., 2008). In addition, the occurrence of GBS within families might also indicate a role for genetic predisposition factors (Geleijns et al., 2004). Thus, gene polymorphisms may be determinants of susceptibility for and severity of clinical neuropathies (Godschalk et al., 2004; Geleijns et al., 2007).

Treatment

Treatment of GBS consists of either intravenous administration of large amounts of human immunoglobulin (IVIg) or plasma exchange. Both treatments shorten the time of recovery and limit the peak symptoms (Lunn and Willison, 2009; Winer, 2008). They are equally efficient and IVIg treatment has become standard in the last few years (Van Doorn et al., 2008; Hughes et al., 2007). Around 10% of the patients still dies from respiratory failure, pulmonary emboli, or co-infection and around 20% has residual disability. Although the disease has an immunological aetiology, steroids are ineffective (which is unusual), and can even slow down recovery (Winer, 2008; Hughes et al., 2007).

High-dose IVIg may have multiple sites of action, such as interference with T-cells and B-cells, cytokine inhibition, and neutralization and catabolism of (auto-) antibodies to gangliosides, thereby preventing complement activation and subsequent pathophysiological effects (Stangel and Pul, 2006; Jacobs et al., 2003). Plasma exchange may act by removing immune complexes, cytokines, complement, and autoantibodies from the patient's plasma. Although both treatments ameliorate the symptoms and possibly shorten the time of recovery in most patients, these therapies are not perfect yet. Plasma exchange is a difficult procedure requiring specially trained staff and it is associated with adverse effects leading to discontinuation of the therapy in 10 – 14 % of cases in various trials (Shahar, 2006; Raphaël et al., 2002; Hughes et al., 2007). IVIg is a blood product from many donors (at least 1000) and carries the risk of containing unknown pathogenic factors. Also IVIg can lead to adverse effects during treatments, though in controlled trials less complications were found in patients treated with IVIg than in patients treated with plasma exchange (Hughes et al., 2006). Both therapies are extremely expensive, and therefore more effective, safer and cheaper therapies are required (Hughes et al., 2006; Hughes et al., 2007).

* CD1 molecules are glycoproteins that capture and present glycolipids to antigen-specific T cells.

Mouse models

Ganglioside knockout mice

Genetically modified mice are powerful tools for investigating fundamental and neurophysiological roles of gangliosides. Several mouse models are engineered in which one or more of the ganglioside synthase genes are disrupted. This eliminates the gangliosides downstream in the biosynthesis pathway (Figure 1.9). The remaining ones are generally upregulated (Takamiya et al., 1996; Okada et al., 2002; Liu et al., 1999). Therefore the total amount of gangliosides (at least in whole brain) in these knockout mice is at the same level as in their wildtype littermates (Kawai et al., 2001; Chiavegatto et al., 2000).

In 1996 a first ganglioside *null*-mutant mice was generated, by deleting the gene for β 1,4-*N*-acetylgalactosaminyltransferase (GalNAc-T or GM2synthase), which is an essential enzyme in the ganglioside biosynthesis. The GM2synthase-knockout (GM2s-KO) mice only express the *simple* series of gangliosides and lack all the complex gangliosides, since these are synthesized via the eliminated enzyme (Takamiya et al., 1996). Though males are infertile due to an impaired transportation of testosterone (Sandhoff et al., 2005), the mice otherwise seem healthy at young age. At older age however, neurodegenerative changes become apparent and mice display whole body tremor and catalepsy. They also show deficits in balance, reflexes, muscle strength and motor coordination and these functional impairments are of progressive nature (Chiavegatto et al., 2000; Ma et al., 2003; Sheikh et al., 1999; Sugiura et al., 2005). Furthermore it was reported that the loss of complex gangliosides results in disrupted paranodal junctions near the nodes of Ranvier and in an impaired integrity of the axonal membrane (Susuki et al., 2007a). Although the pathological effects become more prominent with age, lifespan is normal (Takamiya et al., 1996).

The b- and c-series gangliosides carry a pair of α 2,8-linked sialic acids on the internal galactose of the gangliotetraosyl core. GD3synthase knockout (GD3s-KO) mice have a disrupted *Siat8a* gene which encodes for the enzyme (α 2,8sialyltransferase) that is responsible for the addition of the second sialic acid. These knockout mice are only able to express the O- and a-series gangliosides. The mice are viable and both male and female are fertile. They display no apparent neurological phenotype. A role for b- and c-series gangliosides in neuronal repair mechanisms was suggested from axotomy studies where it was found that GD3s-KO mice had reduced regeneration of hypoglossal nerves (Handa et al., 2005; Okada et al., 2002).

By crossbreeding the above mutants, GM2*GD3synthases double knockout (dKO) mice were created. These mice express only ganglioside GM3 and its precursors and, surprisingly, are still viable. Two independent lines have been published and both lines show a spontaneous adult lethal phenotype (Kawai et al., 2001; Inoue et al., 2002). One strain is susceptible to lethal audiogenic seizures, which might explain this sudden death phenotype (Kawai et al., 2001). In the other strain, refractory skin lesions on the face were reported at or after 25 weeks of age. It was suggested to be the result of over-scratching due to a reduced sensory function (Inoue et al., 2002). Furthermore peripheral nerve degeneration was observed in relatively young mice (Inoue et al., 2002).

Another mutant is the GM3synthase knockout mouse that only expresses the O-series gangliosides. These mice are viable and fertile, and do not show major neurological

Animal models of GBS

For GBS and its variants, so far no perfect *in vivo* animal model exist that mimics all features of the disease. However, there are some partial models for research (Meyer Zu Hörste et al., 2008). In 2001, a rabbit model for AMAN was established, in which GM1 or bovine brain ganglioside is subcutaneously administered for 2 to 3 weeks during which the animals develop limb weakness. The disease shows an acute onset and is monophasic. Wallerian degeneration without demyelination, together with macrophage recruitment to the axons and IgG deposition on root nerves are pathological findings in this model, consistent with human AMAN (Yuki et al., 2001; Susuki et al., 2003; Phongsisay et al., 2008; Susuki et al., 2007b). Experimental autoimmune neuritis (EAN) in rats is a preclinical model of AIDP. Rats are immunized with bovine proteins from PNS (e.g. myelin) in combination with complete Freund's adjuvant. They develop severe motor deficits, associated with demyelination and neural degeneration. EAN is monophasic and indications for allodynia in the pre-onset phase were found, a feature which also can be seen in GBS patients (Mangano et al., 2008; Luongo et al., 2008; Sarkey et al., 2007; Cosi and Versino, 2006).

Immunization of mice with anti-GD1a antibodies in combination with a sciatic nerve crush model was used to directly study ganglioside involvement in axonal regeneration, which may be an important recovery factor in GBS. The antibodies were found to inhibit regeneration (Lehmann et al., 2007). Furthermore, in rabbits it was found that *C. jejuni* LPS from GBS patients induced anti-GM1 IgG (Ang et al., 2000). However, in both these mouse and rabbit models no clinical signs of GBS (i.e. paralysis) were observed. In chapter 6 a newly generated mouse model is described, in which mice were passively immunized with anti-GQ1b antibodies and received additional human complement through intraperitoneal injection. Acute-onset respiratory paralysis was observed in this model (Halstead et al., 2008).

Aims and outlines of this thesis

The first objective of this thesis was to characterize the contribution of specific ganglioside subsets compositions to neurotransmitter release at the NMJ; second, to further elucidate the pathophysiological role of anti-ganglioside antibodies at the NMJ; and third, to generate an *in vivo* mouse model for GBS and to study the therapeutic potency of (experimental) complement inhibitors.

Chapters 2 and 3 focus on the physiological and the pathophysiological roles of gangliosides at the NMJ, respectively:

Chapters 2.1 and 2.2 describe the studies into the roles of gangliosides in synaptic function at the NMJ in ganglioside-knockout mice. Chapter 2.1 focuses on the GD3s-KO mouse, expressing only the O- and a-series gangliosides, as well as on the GM2*GD3s-dKO mouse, lacking all gangliosides except GM3. In chapter 2.2, synaptic transmission at NMJs of aged GM2s-KO and GD3s-KO mice is studied in detail. Old GM2s-KO mice develop motor coordination defects and the question arises whether NMJ dysfunction contributes to this neurological phenotype.

Chapter 2.3 describes a study on the *in vitro* effect of neuraminidase treatment on NMJ function of the dKO mouse. Neuraminidase removes the sialic acid from the single ganglioside left (GM3), so that no gangliosides remain. This enabled us to study the effect of acute, total ganglioside absence on synaptic transmission.

With our increasing knowledge of GBS and its clinical variants, the research now focuses on the involvement of the complement system in the neuropathophysiological features of the disease. Chapter 3.1 outlines the role of complement and complement mediators in motor nerve terminal injury. In chapters 3.2 and 3.3, the involvement of complement in GBS was used to explore new inhibiting therapeutics: a newly developed neuropathic *in vivo* mouse model for MFS was used to test a humanized monoclonal antibody and, furthermore, a tick saliva protein was tested in the *in vitro* model. Both compounds block the complement cascade at the level of C5. In our mouse models the tested therapeutics were able to prevent complement-mediated pathophysiological activities and thereby also prevented structural damage of the NMJ.

In sera of part of the GBS patients, antibodies have been found which target a complex of two gangliosides, but not the individual species. In chapter 3.4 the ability of such sera to induce complement-mediated NMJ damage was explored.

Anti-GM1 ganglioside antibodies show highly inconsistent abilities for binding at neuronal membranes and inducing pathogenic effects. This can be attributed to the fact that GM1 in the live membrane is cryptic for a proportion of the anti-GM1 ganglioside antibodies due to a masking effect of neighbouring gangliosides. In chapter 3.5 it is explored how the level of complexity of ganglioside membrane topology accounts for inter-antibody variability in their experimental neuropathophysiological effects.

The results of chapter 2 and 3 are summarized and discussed in general in chapter 4.

CHAPTER 2

Gangliosides and neuromuscular synaptic function

CHAPTER 2.1

Neuromuscular synaptic function in mice lacking major subsets of gangliosides

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Published in Neuroscience 126 (2008) 885 – 897

Abstract

Gangliosides are a family of sialylated glycosphingolipids enriched in the outer leaflet of cell neuronal membranes, in particular at synapses. Therefore, they have been hypothesized to play a functional role in synaptic transmission. We have measured in detail the electrophysiological parameters of synaptic transmission at the neuromuscular junction (NMJ) *ex vivo* of a GD3synthase knockout mouse, expressing only the O- and a-series gangliosides, as well as of a GM2*GD3synthase double-knockout (dKO) mouse, lacking all gangliosides except GM3. No major synaptic deficits were found in either *null*-mutant. However, some extra degree of rundown of acetylcholine release at high intensity use was present at the dKO NMJ and a temperature-specific increase in acetylcholine release at 35 °C was observed in GD3synthase knockout NMJs, compared to wildtype. These results indicate that synaptic transmission at the NMJ is not crucially dependent on the particular presence of most ganglioside family members and remains largely intact in the sole presence of GM3 ganglioside. Rather, presynaptic gangliosides appear to play a modulating role in temperature- and use-dependent fine-tuning of transmitter output.

Acknowledgements

This work was sponsored by grants from the Prinses Beatrix Fonds (#MAR04-0213) and from the Wellcome Trust (060349, 077041). We thank Marloe Pijnacker for excellent caretaking of the breeding of the mouse strains.

Introduction

Neuronal membranes contain high levels of gangliosides (Ledeen, 1985), which are a diverse family of sialylated glycosphingolipids (Figure 2.1; Svennerholm, 1994; Ngamukote et al., 2007). *Complex* gangliosides GM1, GD1a, GD1b and GT1b are the major species in central and peripheral nervous tissue (Tettamanti et al., 1973; Gong et al., 2002). *Simple* gangliosides (e.g. GM3 and GD3) are relatively abundant in early embryonic brain but decrease rapidly in later stages (Ngamukote et al., 2007).

Gangliosides are components of membrane lipid rafts and are thought to play roles in modulation of membrane-bound enzymes, ion-channel kinetics, cell-adhesion, neuritogenesis, cell-signaling and membrane stability and maintenance (Yates and Rampersaud, 1998; Hakomori, 2003; Hashiramoto et al., 2006; Sohn et al., 2006; Ledeen and Wu, 2006b; Sonnino et al., 2007; Susuki et al., 2007b; Wu et al., 2007).

Importantly, gangliosides are involved in neurological disease. Anti-ganglioside antibodies have been shown in the Guillain-Barré syndrome (GBS) (Willison and Yuki, 2002; Ang et al., 2004), where anti-GM1 antibodies are mainly associated with motor variants of the disease and anti-GQ1b antibodies with the Miller Fisher syndrome variant (Willison and Yuki, 2002; Ang et al., 2004). Ganglioside metabolism is disturbed in an infantile epilepsy syndrome (Simpson et al., 2004), in Sandhoff's disease (Liu et al., 1999), and possibly in Huntington's disease (Desplats et al., 2007) and multiple sclerosis (Marconi et al., 2006). Gangliosides can also function as cell surface receptors for microbial toxins (Fishman, 1982; Bullens et al., 2002).

Gangliosides contain negatively charged sialic acid residues that contribute to the surface charge of membranes and thereby potentially modulate H^+ and Ca^{2+} homeostasis as well as voltage-gated ion-channel functioning. In addition, they influence membrane viscosity in a temperature-dependent way and thereby indirectly modulate ion-channel activities (Kappel et al., 2000). Gangliosides in the vicinity of ion-channels or -pumps may directly influence their kinetics and function (Wang et al., 1999a; Ledeen and Wu, 2006a). Furthermore, they may influence intracellular Ca^{2+} homeostasis (Wu et al., 2005). Recent functional studies using anti-ganglioside antibodies suggest a relationship between gangliosides and voltage-gated Ca^{2+} channels (Ortiz et al., 2001; Santafé et al., 2005; Nakatani et al., 2007b).

In the light of their known effects on ion-channels and their particular abundance in synaptic regions, gangliosides are thought to play a role in neurotransmitter release, which is critically dependent on presynaptic ion-channel function (Wieraszko and Seifert, 1985; Ramirez et al., 1990; Egorushkina et al., 1993; Takamiya et al., 1996; Tanaka et al., 1997; Furuse et al., 1998; Ando et al., 1998; Meir et al., 1999; Chiavegatto et al., 2000; Bullens et al., 2002; Hakomori, 2003; Proia, 2003). Several important proteins of the release machinery co-localize with gangliosides within lipid rafts (Chamberlain et al., 2001; Lang et al., 2001; Taverna et al., 2004; Salaün et al., 2004).

β 1,4-GalNAc-transferase (EC 2.4.1.92) knockout (GM2s-KO) mice lack complex gangliosides (Figure 2.1; Takamiya et al., 1996; Sheikh et al., 1999) and develop sensory and motor coordination defects upon ageing (Chiavegatto et al., 2000; Sugiura et al., 2005). Previously we investigated synaptic transmission in young GM2s-KO mice at NMJs *ex vivo* (Bullens et al., 2002). Surprisingly, we found that complex gangliosides were redundant for acetylcholine (ACh) release at room

temperature. However, reduced release was found at 17 °C, suggesting that complex gangliosides are involved in temperature-stabilization of synaptic transmission.

Two additional ganglioside-deficient mice have been generated: 1) a GD3synthase knockout (GD3s-KO) mouse (Okada et al., 2002), which lacks the gene coding for α 2,8-sialyltransferase (EC 2.4.99.8) and only expresses the O- and a-series gangliosides and 2) a GM2*GD3synthase double knockout (dKO) mouse (Kawai et al., 2001; Inoue et al., 2002), which lacks the genes coding for both β 1,4-GalNAc-transferase and α 2,8-sialyltransferase, thereby only expressing GM3 ganglioside (Figure 2.1). GD3s-KO mice are viable and fertile and show no overt phenotype, while dKO mice display sudden death starting from about 7-12 weeks of age. Here, we studied the roles of specific ganglioside subsets in neurotransmission by characterizing neuromuscular function in these two *null*-mutants.

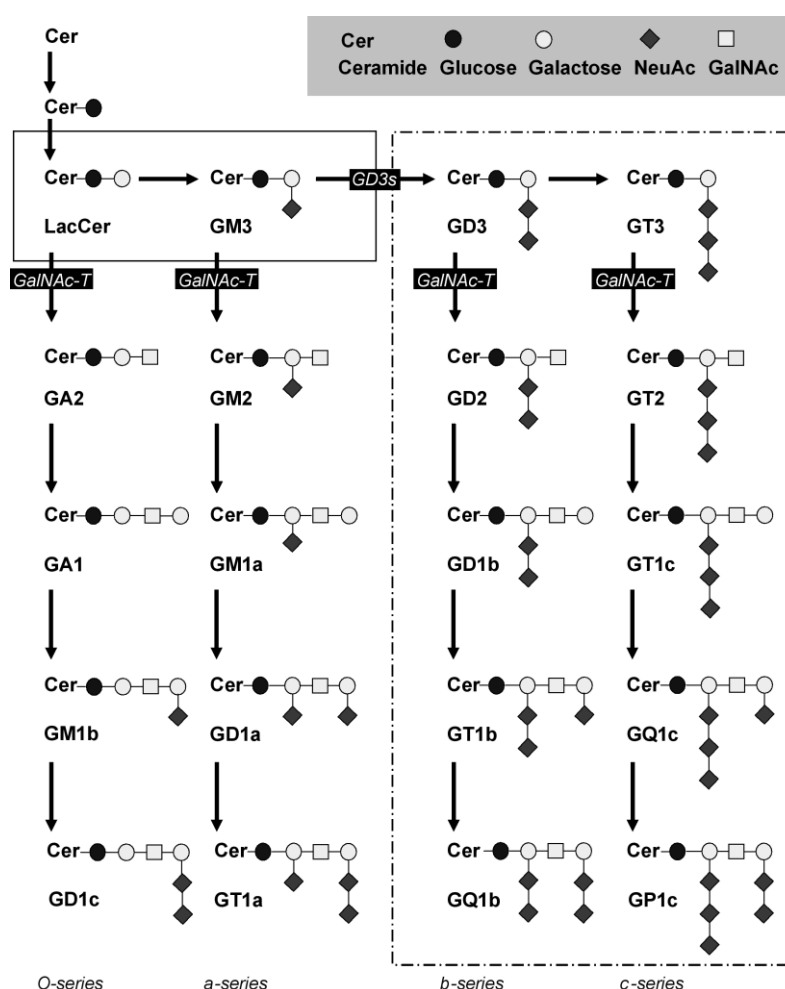


Figure 2.1. Synthesis of the ganglioside family

Ganglioside nomenclature is according to Svennerholm (Svennerholm, 1994). Membranes of wildtype mice contain all the gangliosides. GD3s-KO mice lack the GD3s gene, which results in the absence of all the gangliosides within the dashed rectangle (b- and c-series). dKO mice lack both the GM2synthase (GalNAc-T) and the GD3synthase genes, leaving expression of only GM3 and its precursor lactosylceramide (LacCer), shown within the continuous rectangle. NeuAc: neuraminic acid (or sialic acid); GalNAc-T: N-acetylgalactosamine transferase; GD3s: GD3synthase. Arrows represent the stepwise biosynthesis through glycosyltransferases.

Material and methods

Mice

We used male and female GD3s-KO mice (Okada et al., 2002) for experiments. To generate the dKO mice, homozygous female GM2s-KO mice (Takamiya et al., 1996) were crossbred with male homozygous GD3s-KO mice. Their double heterozygous progeny was then intercrossed to generate homozygous dKO mice. Genotyping was performed as described (Takamiya et al., 1996; Inoue et al., 2002). Male and female dKO mice were used in the experiments. Wildtype (WT) mice were used as controls. The mice were 6-13 weeks of age. Body weights of WT, GD3s-KO and dKO mice used were 20.3 ± 1.0 , 21.1 ± 0.8 and 21.5 ± 0.8 g, respectively. All animal experiments were carried out according to Dutch law and Leiden University guidelines and the USA National institute of Health recommendations for the humane use of animals. All efforts were made to minimize the number of animals used and their suffering.

In vivo neuromuscular function tests

The inverted screen hanging test was used to assess fatigability of limb muscles as described before (Kaja et al., 2007a). The test ended upon falling or completing the maximum hanging time which was set at 300 s.

Muscle strength was assessed using a grip strength meter (type 303500, Technical and Scientific Equipment GmbH) and essentially performed as described (Kaja et al., 2007a). As grip strength the peak force value was taken of a pull measured by the grip strength meter. Each trial consisted of at least 10 pullings and the averaged value was used for statistical analysis. Values were normalized to the body weights of mice.

Respiratory rate and volume were assessed with non-invasive whole-body plethysmography (RM-80, Columbus Instruments). The signal was digitized using a Digidata 1440A interface (Axon Instruments/Molecular Devices) and analyzed with the event detection feature of Clampfit 9.2 (Axon Instruments/Molecular Devices).

In vitro electrophysiology at the NMJ

Mice were killed by CO₂ asphyxiation. Left and right hemi-diaphragms were dissected with their phrenic nerve attached and mounted in standard Ringer's medium (119 mM NaCl, 4.5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 23 mM NaHCO₃, 11 mM glucose, pH 7.4) at room temperature, pre-gassed with 95% O₂ / 5% CO₂.

Intracellular recordings of miniature endplate potentials (MEPPs) and endplate potentials (EPPs) in the NMJ were made using a glass micro-electrode (10-20 MΩ, filled with 3 M KCl) connected to a Geneclamp 500B (Axon Instruments/Molecular Devices) for amplifying and filtering (10 kHz low-pass) of the signal. The signal was digitized using a Digidata 1322A interface (Axon Instruments/Molecular Devices) and analyzed using Clampfit 9.2 (Axon Instruments/Molecular Devices) and Mini Analysis 6.0.3 (Synaptosoft). Muscle action potentials were eliminated by using the selectively skeletal muscle Na⁺ channel blocker, μ-conotoxin GIIIB (3 μM) (Scientific Marketing Associates). To record EPPs, the phrenic nerve was stimulated at multiple frequencies using a bipolar platinum electrode. The mean EPP and MEPP amplitudes at each NMJ were normalized to -75 mV, with the reversal potential for ACh-induced current assumed to be 0 mV (Magleby and Stevens, 1972). In order to calculate the quantal content for each NMJ, the mean amplitude of the 30 EPPs recorded at low

rate (0.3 Hz) stimulation was corrected for non-linear summation (McLachlan and Martin, 1980) and the normalized and corrected mean EPP amplitude was divided by the normalized mean MEPP amplitude (calculated from at least 40 MEPPs sampled). The quantal content is the number of ACh quanta that is released upon a single nerve impulse.

MEPPs were also recorded after addition of hypertonic medium (0.5 M sucrose Ringer's medium), in order to estimate the pool of ACh vesicles ready for immediate release (Stevens and Tsujimoto, 1995; Varoqueaux et al., 2005). In some experiments we tested the effect on EPPs and MEPPs of 200 nM of the $\text{Ca}_v2.1$ Ca^{2+} blocker ω -agatoxin-IVA (Scientific Marketing Associates) and 10 μM of the Ca_v1 blocker nifedipine (Sigma-Aldrich).

Temperature of the bath medium was controlled using a Peltier device placed around the recording bath and adjusted by varying the DC output of a power supply (Delta Elektronika). A miniature probe connected to a digital thermometer was used to monitor the temperature. Bath temperature was held at 24-26 °C, unless stated otherwise.

Electrophysiological data are presented as group mean $\pm$ SEM of the mean muscle values calculated from the mean NMJ values. At least 10 NMJs were sampled per muscle per experimental condition.

Statistical analysis

Statistical differences between group means were analyzed with an unpaired Student's *t*-test or an analysis of variance (ANOVA), with Bonferroni post hoc testing, wherever appropriate.

Results

***In vivo* neuromuscular tests**

Neuromuscular synapse dysfunction may lead to respiration difficulties that are detectable in whole-body plethysmography (Halstead et al., 2008). Upon such assessment we found in both the GD3s-KO and dKO mice a lower respiration rate than in WT mice (WT 424 ± 20 ; GD3s-KO 298 ± 10 ; dKO 307 ± 14 min^{-1} ; $n=8-20$ mice, $p<0.01$; Figure 2.2A, C). The positive peak amplitude of the measured signal, reflecting tidal volume, was ~35% increased in dKO mice (WT 12.2 ± 0.7 ; GD3s-KO 12.2 ± 0.4 ; dKO 16.4 ± 0.6 mV; $p<0.01$; Figure 2.2B, C). When the signals were normalized to body weight this difference became somewhat smaller but remained statistically significant (WT 0.65 ± 0.03 ; GD3s-KO 0.59 ± 0.02 ; dKO 0.77 ± 0.03 mV per g body weight; $p<0.01$ dKO vs. GD3 and $p<0.05$ dKO vs. WT). Figure 2.2C shows typical examples of respiration traces recorded.

GD3s-KO mice showed no overt neuromuscular phenotype. In 6 of the 14 dKO mice used, however, we observed symptoms of weakness and/or uncoordinated movement (especially of hind legs). Although grip strength testing indicated normal muscle strength in dKO mice (~ 6 g/g body weight for each strain; $p=0.30$, $n=7-20$ mice; Figure 2.2D), they performed worse on the inverted mesh (hanging times: WT 300 ± 0 ; GD3-KO 277 ± 16 ; dKO 178 ± 28 s; $p<0.05$, $n=5-20$ mice; Figure 2.2E). Symptomatic dKO mice had shorter ($p<0.05$) hanging times (111 ± 39 s) than non-

symptomatic dKO mice (228 ± 30 s), but did not differ from each other with respect to the other *in vivo* tests.

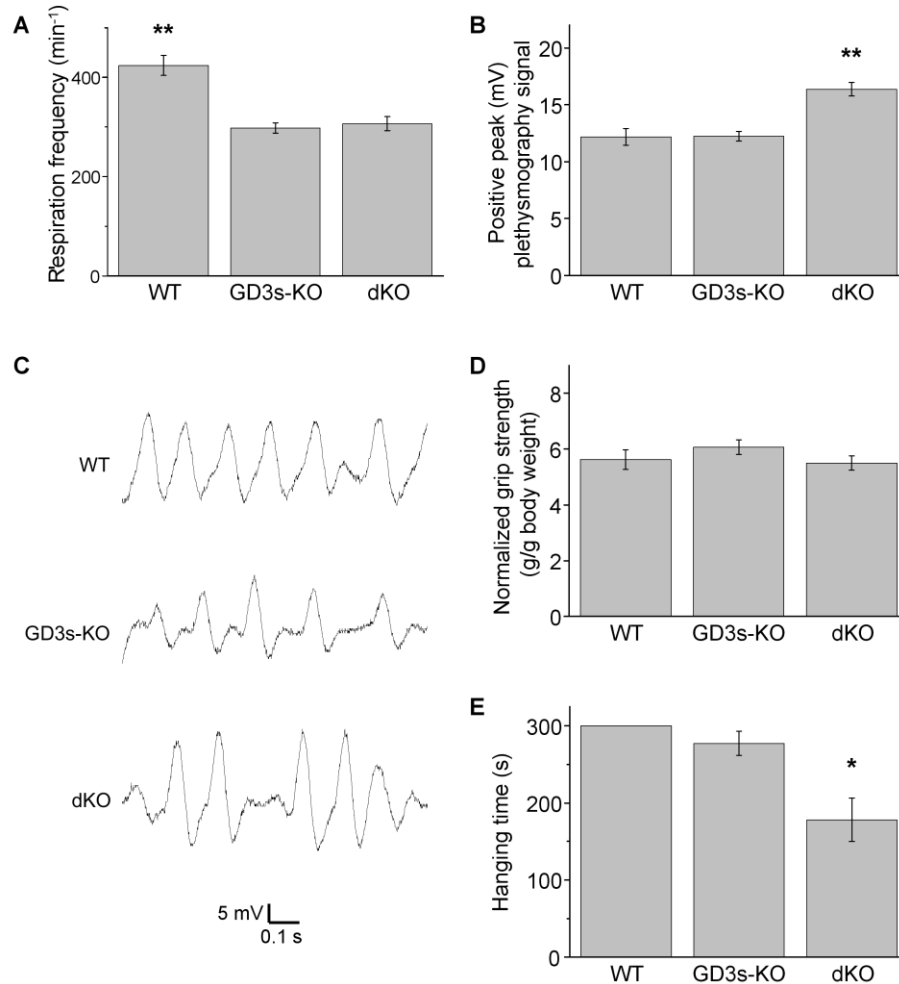


Figure 2.2. *In vivo* assessment of neuromuscular functioning

Mouse respiration was characterized with non-invasive plethysmography ($n = 8-20$). **A.** Respiration frequency was ~40% higher in WT mice than in the other two groups. **B.** The positive peak of the plethysmography signal (reflecting tidal volume) was ~35% higher in dKO mice, compared with WT and GD3s-KO. **C.** Typical examples of respiration traces recorded. **D.** Grip strength was averaged for ten trials and normalized to the body weights of the mice; all groups pulled ~6 g per gram bodyweight ($n=7-20$). **E.** Hanging times in the inverted screen test. A maximum hanging time of 300 s was chosen. dKO mice had considerably shorter hanging times than WT and GD3s-KO mice ($n = 5-20$).

* $p < 0.05$; ** $p < 0.01$

Normal basic synaptic electrophysiology in GD3s-KO and dKO mice

Spontaneous unquantal transmitter release was measured at the diaphragm NMJ. No statistically significant differences were found between genotype groups in MEPP amplitude (~0.9 mV; $p = 0.43$, Figure 2.3A, E) and MEPP frequency (~1.3 s⁻¹; $p = 0.10$, Figure 2.3B). The 0.3 Hz nerve stimulation-evoked transmitter release resulted in equal EPP amplitudes for the three groups (~25 mV; $p = 0.45$, Figure 2.3C, F). The

delay between nerve stimulus and start of the EPP was somewhat longer in dKO preparations (WT 1.74 ± 0.09 ; GD3s-KO 1.84 ± 0.05 ; dKO 2.16 ± 0.11 ms; $p < 0.05$ dKO vs. WT). Quantal content was calculated and appeared not statistically significantly different between groups although there was a tendency towards reduction in the dKO NMJ (WT 47.0 ± 2.7 ; GD3s-KO 46.1 ± 1.9 ; dKO 40.3 ± 1.0 ; $p = 0.27$, Figure 2.3D).

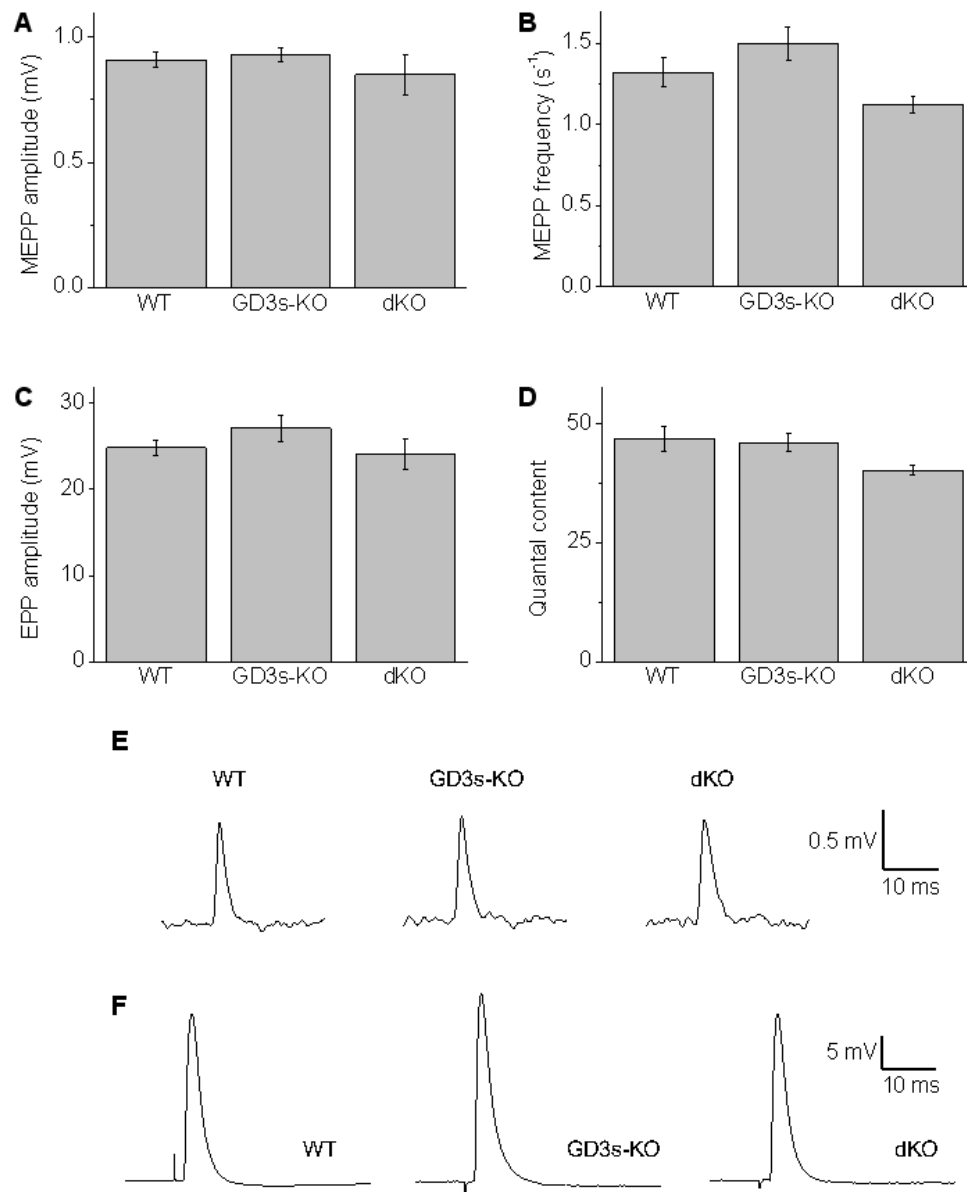


Figure 2.3. Basic ACh release parameters at the NMJ

Ex vivo electrophysiological measurements at NMJs of phrenic nerve-diaphragm preparations were performed at 24–26 °C in standard Ringer's medium. Each genotype group consisted of at least 5 mice. **A.** Uniquantal size measured as MEPP amplitude. **B.** Spontaneous uniquantal ACh release measured as MEPP frequency. **C.** EPP amplitude at 0.3 Hz stimulation. **D.** Calculated quantal content of EPP at 0.3 Hz nerve stimulation. **E.** Typical examples of MEPP recordings. **F.** Typical examples of EPP recordings. No statistically significant differences between genotypes were observed.

ACh release at the NMJ is almost completely mediated by Ca^{2+} flux through $\text{Ca}_v2.1$ (P/Q-type) channels. We probed for compensatory contribution of non $\text{Ca}_v2.1$ channels by determining the effect of 200 nM ω -agatoxin-IVA ($\text{Ca}_v2.1$ blocker) and 10 μM nifedipine (Ca_v1 blocker) on 0.3 Hz evoked ACh release at both dKO and GD3s-KO NMJs. ω -Agatoxin readily reduced the quantal content by 95% at NMJs of both genotypes (from 28.4 ± 1.6 to 1.4 ± 0.3 in the dKO and from 44.1 ± 4.8 to 2.1 ± 0.5 in the GD3s-KO; one hemi-diaphragm preparation each, 10-15 NMJs sampled per condition), indicating an almost complete dependence on $\text{Ca}_v2.1$, identical to the reductions we observe routinely at WT NMJs (Kaja et al., 2007a). This seems to rule out any compensatory contribution by other types of Ca_v channels. The Ca_v1 (L-type) channel blocker nifedipine was without effect on the quantal content measured at dKO and GD3s-KO NMJs, excluding compensation by Ca_v1 channels. The quantal content values were 29.9 ± 2.6 before and 31.3 ± 0.3 after nifedipine in the dKO and 44.1 ± 4.8 before and 44.1 ± 4.1 after nifedipine in the GD3s-KO (one hemi-diaphragm preparation each, 10-15 NMJs sampled per condition).

No change in hypertonic shock-induced ACh release

Hypertonic medium (0.5 M sucrose Ringer's) elevated MEPP frequencies to equal levels in the three genotype groups ($\sim 48 \text{ s}^{-1}$; $p=0.48$, $n=3-6$; Figure 2.4). These results suggest an unchanged size of the readily releasable ACh vesicle pool at the NMJ of the two *null*-mutant mice.

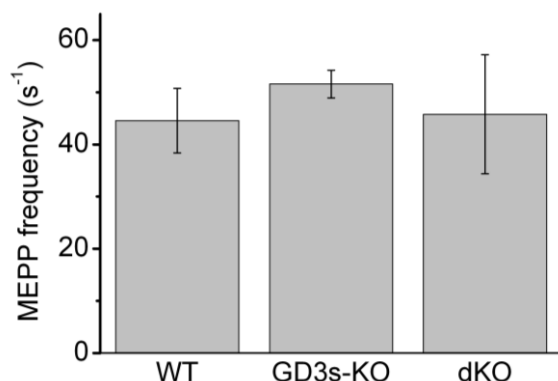


Figure 2.4. Hypertonic medium-evoked ACh release

MEPP frequency in hypertonic medium (0.5 mM sucrose Ringer's). No statistically significant differences between genotypes were observed ($n=3-5$).

More pronounced ACh release rundown at dKO NMJs at high rate nerve stimulation

Some effects of ganglioside composition on transmitter release may only come about at high intensity use of the synapse, stressing the exocytotic molecular machinery. We, therefore, measured EPPs during 1 s high-rate (40 Hz) nerve stimulation trains, which is the approximate physiological firing frequency. At dKO NMJs a modest but statistically highly significant increase in EPP rundown was observed, compared with the other two groups. The EPP rundown plateau levels (the mean of the 21st-35th EPP, expressed as percentage of the first EPP in the train) were: WT 81.2 ± 0.9 ; GD3s-KO 83.0 ± 0.8 ; dKO $75.7 \pm 0.8\%$ ($p<0.01$, $n=11-12$ mice; Figure 2.5A). At 3 Hz no such difference was encountered. The rundown level in all groups was $\sim 83\%$ ($p=0.15$). In a separate experimental series on dKO NMJs we explored the behaviour of ACh release at stimulation frequencies of 3, 30, 40, 50, and 70 Hz. Except for 3 Hz ($p=0.65$), dKO

EPPs ran down to a 5-7% lower level than WT at all stimulation frequencies ($p < 0.01$; Figure 2.5B). Examples of typical EPP rundown traces are shown in Figure 2.5C.

Temperature-dependent changes in transmitter release parameters

We explored a possible temperature-dependent functioning of gangliosides by performing synaptic electrophysiological measurements at 17, 20, 30, and 35 °C in a separate series of experiments (Figure 2.6). No major differences between GD3s-KO, dKO and WT NMJs in the temperature-dependency of synaptic transmission parameters (MEPP amplitude and frequency, EPP amplitude and quantal content) were observed within this temperature range, other than a somewhat increased MEPP amplitude at GD3s-KO NMJs at 17 °C (~30%, $p < 0.05$; Figure 2.6A) and an increase of quantal content in GD3s-KO NMJs (~40%, $p < 0.05$), compared with WT, at 35 °C (Figure 2.6D). The rundown of EPPs at 40 Hz stimulation at dKO NMJs was more pronounced than in GD3s-KO and WT, over the whole temperature range, although only statistically significantly at 25 °C (as described above), 30 and 35 °C test temperatures ($p < 0.05$; Figure 2.6E). Because the extent of this extra EPP rundown was similar (~25%) at the several temperatures, it can be concluded that the three genotype groups show a similar temperature-dependency for this parameter. The 3 Hz EPP rundown at both dKO and GD3s-KO NMJs were not statistically significantly different from WT at all tested temperatures, although there was a tendency of increased rundown at dKO NMJs. At 17 and 30 °C, 3 Hz EPP rundown at dKO NMJs was somewhat more pronounced than at GD3s-KO NMJs ($p < 0.05$; Figure 2.6F).

Influence of external Ca^{2+} concentrations on transmitter release

Besides our basic measurements in 2 mM extracellular Ca^{2+} , low (0.2 mM) as well as high (5 mM) Ca^{2+} extracellular medium was applied to explore the Ca^{2+} -dependency of synaptic transmission in the GD3s-KO and dKO mice. MEPP amplitude was ~0.83 mV for each strain at each Ca^{2+} concentration (Figure 2.7A). MEPP frequency (Figure 2.7B), EPP amplitude (Figure 2.7C) and quantal content (Figure 2.7D) in WT NMJs were, as expected, steeply dependent on Ca^{2+} . However, we observed no different values of these parameters at GD3s-KO and dKO NMJs, compared to WT, at all Ca^{2+} concentrations, showing unaltered Ca^{2+} -sensitivity of these parameters. As observed at 2 mM Ca^{2+} , the 40 Hz EPP rundown level at dKO NMJs in the presence of 5 mM Ca^{2+} (71.3%) was lower than that in WT (79.8%) and GD3s-KO (78.4%) (Figure 2.7E; $p < 0.01$). At low Ca^{2+} and 40 Hz stimulation, WT EPPs were potentiated to a plateau level of ~150% of the first EPP. GD3s-KO and dKO NMJs showed similar EPP potentiation ($p = 0.83$, Figure 2.7E). Example traces of the EPP profiles at 40 Hz stimulation at 0.2 mM Ca^{2+} are shown in Figure 2.7F. EPPs at dKO NMJs at 3 Hz stimulation at 5 mM Ca^{2+} , but not at 0.2 and 2 mM, showed a slightly lower EPP rundown level (79.5%) than at WT (84.8%) and GD3s-KO (85.1%) NMJs (Figure 2.7G, $p < 0.01$). We assessed 25 ms paired-pulse facilitation by comparing the first and second EPP of 40 Hz trains, but found no statistically significant differences between GD3s-KO, dKO and WT NMJs at any of the Ca^{2+} concentrations tested (Figure 2.7H).

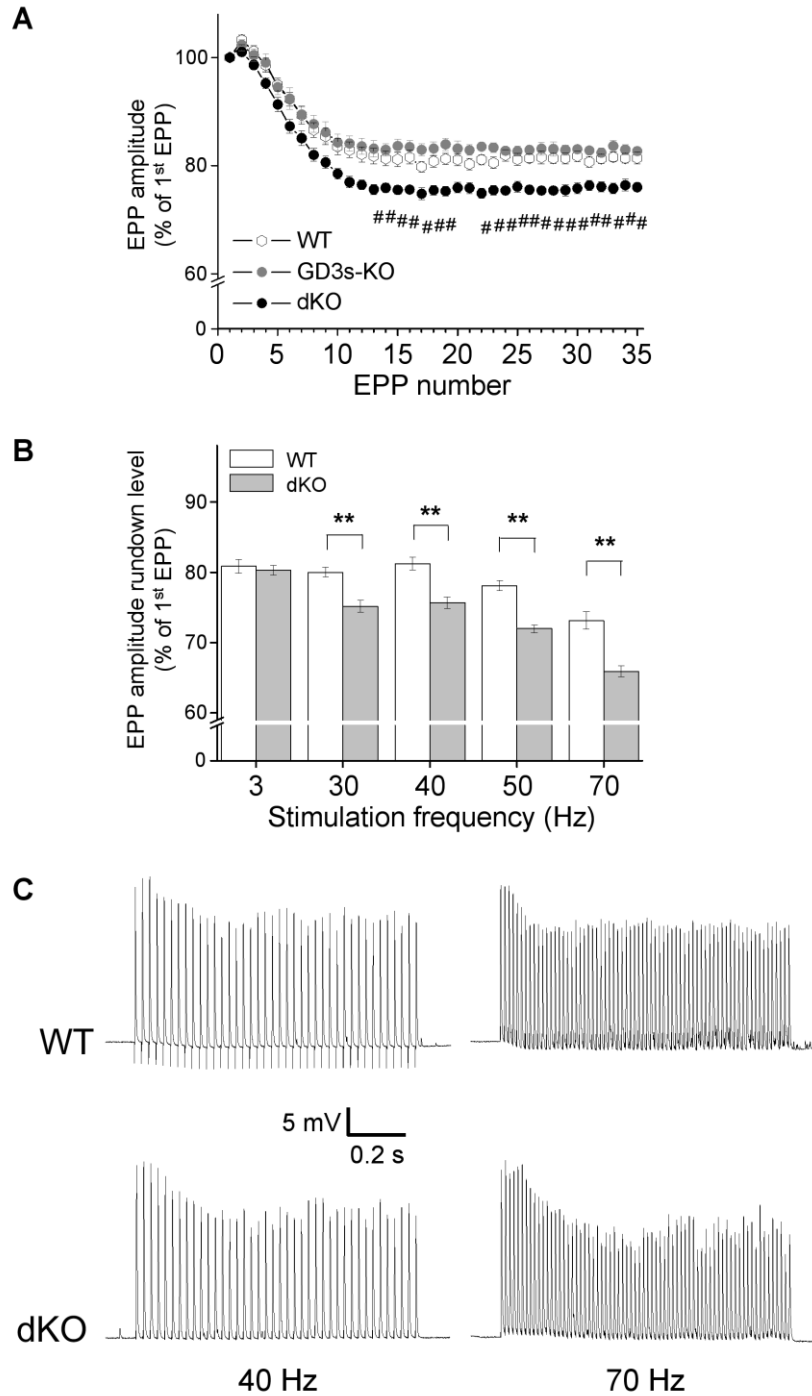


Figure 2.5. Evoked ACh release at high rate stimulation frequencies

A. Mean EPP rundown profile at 40 Hz stimulation. Each EPP in the trains is expressed as percentage of the first EPP. dKO EPPs ran down more pronouncedly than WT and GD3s-KO EPPs ($p < 0.01$; $n = 11-12$). **B.** Rundown level is expressed as the ratio of the mean amplitude of the plateau phase of the train (21st-35th EPP) and the amplitude of the first EPP and was measured at several stimulation frequencies for WT and dKO mice. dKO NMJs displayed lower rundown levels at all frequencies but 3 Hz ($p < 0.01$, $n = 7-12$). **C.** Typical traces of EPP amplitude rundown profiles at 40 and 70 Hz stimulation for WT and dKO NMJs.

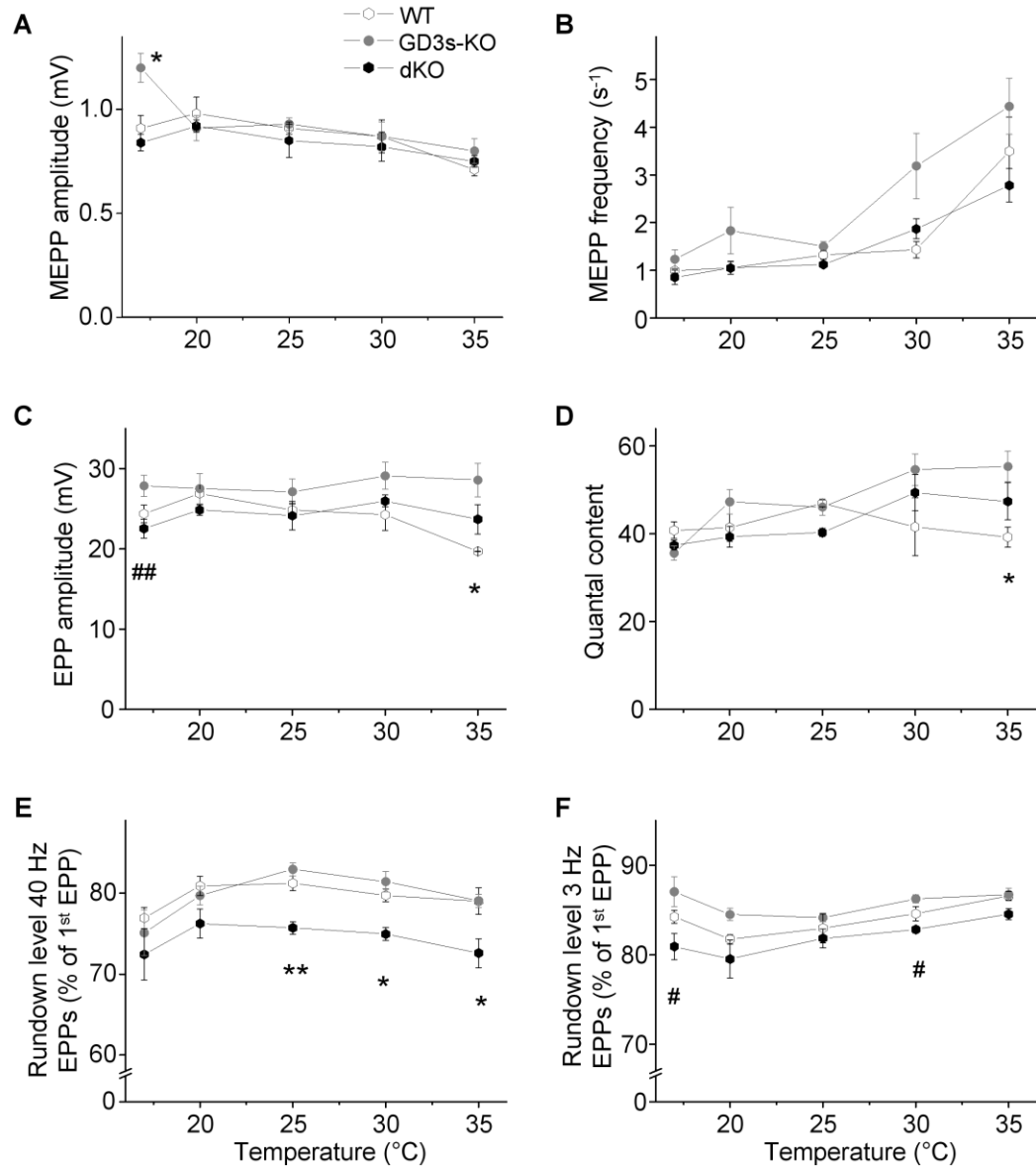


Figure 2.6. Temperature-dependency of ACh release parameters

Electrophysiological measurement of ACh release at diaphragm NMJs at different bath temperatures. Each genotype group consisted of 4-6 mice. **A.** MEPP amplitude at GD3s-KO NMJs was higher than at WT and dKO NMJs at 17 °C. **B.** No statistically significant differences between genotypes in spontaneous ACh release measured as MEPP frequency at all temperatures. **C.** Differences between genotypes in evoked EPP amplitude at 0.3 Hz nerve stimulation were found only at 17 and 35 °C (^{##} $p < 0.01$ for GD3s-KO vs. dKO and $*p < 0.05$ for WT vs. GD3s-KO, respectively). **D.** The quantal content at GD3s-KO NMJs was higher than WT, only at 35 °C ($*p < 0.05$). **E.** dKO mice have lower rundown levels than GD3s-KO and WT mice at > 20 °C. EPP rundown level is expressed as the ratio of the mean amplitude of the plateau phase of the train (21st-35th EPP) and the amplitude of the first EPP at 40 Hz stimulation ($*p < 0.05$; $**p < 0.01$). **F.** dKO and GD3s-KO EPP rundown level at 3 Hz stimulation did not differ from WT at all measured temperatures. Between each other they differed in this parameter at 17 and 30 °C ($#p < 0.05$).

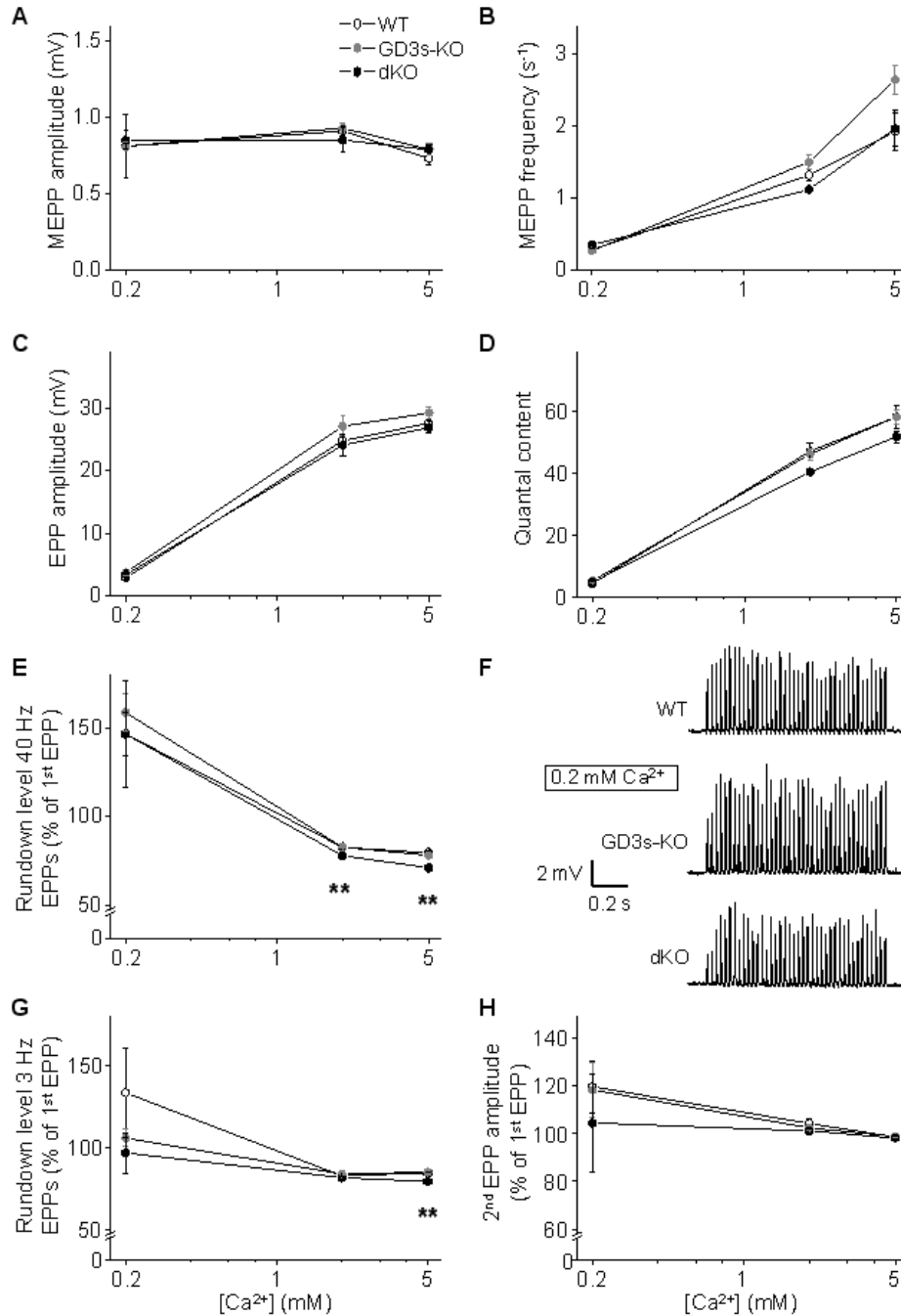


Figure 2.7. Ca^{2+} -dependency of ACh release parameters

Electrophysiological measurement of ACh release at diaphragm NMJs at different extracellular Ca^{2+} concentrations. Each genotype group consisted of 4-6 mice. No differences between genotypes in Ca^{2+} -dependency were found for (A) MEPP amplitude, (B) spontaneous unquantal ACh release, measured as MEPP frequency, (C) evoked EPP amplitude at 0.3 Hz nerve stimulation and (D) calculated quantal content of EPP at 0.3 Hz nerve stimulation. E. Rundown level of EPPs is expressed as the ratio of the mean amplitude of the plateau phase of the train (21st-35th EPP) and the amplitude of the first EPP at 40 Hz stimulation. At 2 and 5 mM Ca^{2+} concentrations we found a lower rundown level at dKO NMJs, compared with WT and GD3s-KO NMJs. F. Typical examples of EPP profiles at 40 Hz stimulation in 0.2 mM Ca^{2+} medium. G. EPP rundown level at 3 Hz stimulation shows a slight, but statistically significant, increase of the rundown at dKO NMJs at 5 mM Ca^{2+} , compared with WT and GD3s-KO. H. No differences between genotypes in paired-pulse (25 ms) facilitation.

** $p < 0.01$

Discussion

We studied the effects of changed ganglioside profiles in neuronal membranes on synaptic transmission by characterizing NMJ function in GD3s-KO mice, lacking b- and c-series gangliosides, and in dKO mice, lacking all ganglioside types except GM3 (Figure 2.1). However, we found no major synaptic deficits in both *null*-mutants. This is quite surprising, in view of studies suggesting a synaptic role for gangliosides on the basis of exogenous ganglioside application (Tanaka et al., 1997; Ando et al., 1998), as well as the reported co-localization in lipid rafts of gangliosides and proteins important for neuroexocytosis (Chamberlain et al., 2001; Lang et al., 2001; Taverna et al., 2004; Salaün et al., 2004). The only changes we observed were some extra degree of rundown of transmitter release at high intensity use at the dKO NMJ and a temperature-specific increase in quantal content at 35 °C in GD3s-KO NMJs, compared with WT. These results indicate that synaptic transmission at the NMJ is not crucially dependent on any particular ganglioside and remains largely intact in the sole presence of GM3 ganglioside.

In vivo neuromuscular analysis

We have performed some orienting *in vivo* characterization of neuromuscular function of the GD3s-KO and dKO mice. Previously we showed that severe paralysis of mouse diaphragm muscle leads to reduced respiration rate and tidal volume in whole-body plethysmography (Halstead et al., 2008). Although we here observed some reduction in respiration rate in both GD3s-KO and dKO mice, tidal volume was not reduced (in fact, there was ~35% increase in the dKO), making severe diaphragm paralysis highly unlikely. Therefore, these changes in respiration patterns probably have a central (possibly synaptic) origin. The reduced hanging time of dKO mice in the inverted mesh test is at least not due to initial forelimb muscle weakness because grip strength testing appeared normal. Although most likely due to central dysfunction, it cannot be excluded that the reduced inverted mesh performance has a fatigue component due to increased rundown of transmitter release at the NMJ (see below).

Basic synaptic transmission

In standard physiological medium at 25 °C the variations in ganglioside composition at GD3s-KO and dKO NMJs did not seriously limit synaptic function. The rate of unquantal ACh release, measured as MEPP frequency, was not different from the WT control NMJs. Unquantal size, measured as MEPP amplitude, as well as the amount of ACh released upon 0.3 Hz nerve stimulation was similar in the three genotypes. These unchanged synaptic function parameters at GD3s-KO and dKO NMJs show that at 0.3 Hz stimulation there is a normal invasion of the nerve impulse into the presynaptic nerve ending and a normal translation of this depolarization into transmitter release. Apparently, the function of voltage-gated Na⁺, K⁺ and Ca²⁺ channels involved in these processes is not impeded by either the loss of b- and c-series gangliosides or by the loss of all gangliosides except GM3. Some degree of ion-channel dysfunction may be present in the dKO phrenic nerve, because we observed ~25% longer delay between nerve stimulus and postsynaptic response in dKO preparations, compared with WT. Although we have not investigated whether this effect is either due to extra synaptic delay or to slower axonal action potential conduction, the observation that GM1 and GD1a are necessary for stability and ion-channel composition of motor nerve nodes of Ranvier (Susuki et al., 2007b) favours

the latter possibility. Our experiments with specific $\text{Ca}_v2.1$ and Ca_v1 Ca^{2+} channel blockers indicated no compensatory contribution by non- $\text{Ca}_v2.1$ Ca^{2+} channels to ACh release at both dKO and GD3s-KO NMJs.

Previously we demonstrated a redundancy for the set of complex gangliosides (i.e. the gangliosides from all series with more than two non-sialic acid sugar residues; Figure 2.1) by finding unaltered basic synaptic function at NMJs of GM2s-KO mice (Bullens et al., 2002). Our present results in dKO and GD3s-KO mice, in combination with this previous study, show that the sole presence of GM3 ganglioside is sufficient to support neurotransmitter release at the NMJ. GM3 is upregulated in dKO whole brains (Kawai et al., 2001) and such an accumulation may also take place at the presynaptic motoneuronal membrane, possibly enhancing compensatory effects of GM3 in supporting neurotransmitter release upon absence of all other types of gangliosides. Alternatively, gangliosides might not influence the function of ion-channels and/or other membrane factors at mouse presynaptic nerve terminal at all. In other organisms, like *Drosophila melanogaster*, functional synapses exist in spite of the inability to produce gangliosides (Roth et al., 1992; Chen et al., 2007a). This shows that gangliosides are not a general prerequisite for synaptic function. However, such absence of a biological role for gangliosides in mammals is rather unlikely in view of the presence of such a highly organized ganglioside synthesis system and, furthermore, the demonstrated severe neurodegeneration in transgenic mice lacking all gangliosides *including* GM3 (Yamashita et al., 2005) and the severe neurological symptoms in human babies with loss-of-function mutated GM3synthase (Simpson et al., 2004). Therefore, it would be of interest to investigate synaptic transmission in GM3synthase *null*-mutant mice.

Increased rundown of high-rate transmitter release at dKO synapses

We observed a more pronounced rundown of high rate (30-70 Hz) nerve stimulation-evoked ACh release at dKO NMJs (EPP rundown plateau levels, expressed as percentage of the first EPP in the trains, were ~6% lower than WT). In contrast, GD3s-KO NMJs showed a 40 Hz rundown level comparable to the WT control. This more pronounced rundown of ACh release at dKO NMJs is, however, not to be expected to negatively impact on successful synaptic transmission. From the mean dKO EPP amplitude of 24 mV and the published safety factor for the mouse NMJ of at least 2.4 (Wood and Slater, 2001) it can be calculated that EPPs of more than 10 mV will result in successful transmission. Even at maximal rundown (to a plateau level of ~66% of their initial value, at 70 Hz stimulation), dKO EPPs would remain ~16 mV, i.e. large enough to trigger an action potential in the muscle fibre. This could explain the absence of overt muscle weakness in the dKO mice. However, it cannot be excluded that a more pronounced EPP rundown underlies the worse performance of dKO mice on the inverted mesh, which tests for fatigue on the longer duration scale (minutes). It may be that the extra EPP rundown in dKO mice became more prominent upon such long-duration and high-intensity use, resulting in subthreshold EPPs.

Rundown of neurotransmitter release is presumably depending on a combination of the inactivation characteristics of presynaptic Ca^{2+} channels and the size and replenishment rate of the pool of releasable ACh vesicles. At least the pool size in dKO motor nerve terminals seems not reduced because MEPP frequency after addition of hypertonic medium, being a measure for pool size (Stevens and Tsujimoto, 1995; Varoqueaux et al., 2005), was not reduced compared with WT. The extra EPP rundown at dKO NMJs was found at most temperatures and Ca^{2+}

concentrations tested here. Previously, we analyzed synaptic function at NMJs of GM2s-KO mice and found some extra rundown of EPP amplitude during high frequency stimulation in particular at 30-35 °C and at a high extracellular Ca^{2+} concentration (Bullens et al., 2002). However, GD3s-KO NMJs did not show extra EPP rundown at any condition. This suggests a role for the O- and/or a-series gangliosides in transmitter release at high frequency nerve stimulation, which could be supported by the finding that in particular GM1 ganglioside influences cellular Ca^{2+} membrane flux and homeostasis (Wu et al., 2004), including stimulation of Ca^{2+} influx in some cell types (Wu et al., 1990). If GM1 would promote Ca^{2+} influx at the motor nerve terminal through slowing down Ca^{2+} channel inactivation this could explain the extra EPP rundown in the dKO NMJs where GM1 is absent. Ion-channel kinetics have been shown to be influenced by gangliosides, either through an electrical effect of the negative charges on their head groups or by an indirect mechanical effect through their ability to control membrane fluidity (Kappel et al., 2000).

Temperature-dependency

Gangliosides have been hypothesized to play a role in thermal stabilization of the neuronal membrane, including adaptation of ion-channel function (Rahmann et al., 1998). We observed a slight tendency of the quantal content at dKO and GD3s-KO NMJs to increase with increasing temperature. At 35 °C this resulted in statistically significantly higher level (~40%) at GD3s-KO NMJs, compared with the WT level which itself remained more or less equal at all temperatures tested. On the basis of this observation it can be hypothesized that gangliosides inhibit transmitter release, e.g. through a (temperature-dependent) effect on activation and/or inactivation of presynaptic voltage-dependent K^+ and/or Ca^{2+} channels. The negative charges on extracellular sialic acid residues of gangliosides contribute to membrane surface charge and may thus influence voltage-dependent properties of ion-channels (Green and Andersen, 1991). For instance, it has recently been shown that removal of sialic acid from the extracellular neuronal membrane by neuraminidase treatment shifts the activation- and inactivation-voltage of Na^+ channels (Isaev et al., 2007), although it remains unclear whether removal of sialic acid from either the surrounding gangliosides or the ion-channel protein itself is causing this effect. The tendency for increased quantal content at 35 °C at dKO compared with WT NMJs indicates that the presence of GM3 alone is not sufficient to keep quantal content at WT level. This may be related to the relatively low level of sialylation of GM3 (only a single sialic acid residue) as compared with other types of gangliosides, because this degree influences the specific effects of gangliosides on ion-channel function (Kappel et al., 2000). An alternative explanation for the tendency of an increase of quantal content at GD3s-KO and dKO NMJs at the higher temperatures tested could be an increased Ca^{2+} availability. Gangliosides have temperature-dependent Ca^{2+} binding sites of which the amount is positively associated with the extent of sialylation (Rahmann et al., 1998). Therefore, loss of sialic acid density in the vicinity of presynaptic voltage-gated Ca^{2+} channels could lead to less adequate Ca^{2+} buffering and thus to increased ACh release. Such an effect seems to occur rather specifically at temperatures around 35 °C, because in our Ca^{2+} variation experiments at 25 °C all GD3s and dKO synaptic parameters except dKO EPP rundown showed normal Ca^{2+} -dependencies.

Conclusion

In conclusion, our results show that most types of gangliosides are not crucially important for synaptic transmission at the mouse NMJ but, rather, have a role in temperature- and use-dependent fine-tuning of transmitter release level. It remains to be directly shown whether the remaining (and possibly upregulated) presence of the *simple* ganglioside GM3 at dKO membranes explains the partial redundancy of all other gangliosides in neurotransmission.

CHAPTER 2.2

Neuromuscular synaptic transmission in aged ganglioside-deficient mice

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Neurobiology of Aging (2009), in press

Abstract

Gangliosides are sialylated glycosphingolipids that are present in high density on neuronal membranes, especially at synapses, where they are assumed to play functional or modulating roles. Mice lacking GM2synthase express only the simple gangliosides GD3 and GM3 and develop progressive motor behaviour deficits upon ageing, apparently due to failing complex ganglioside-dependent maintenance and/or repair processes or, alternatively, toxic GM3/GD3 accumulation. We investigated the function of neuromuscular junctions (NMJs) of aged (>9 months-old) GM2synthase *null*-mutant mice, because synaptic dysfunction might develop with age and could potentially contribute to the late-onset motor phenotype. In addition, we studied NMJs of old mice lacking GD3synthase (expressing only O- and a-series gangliosides), which do not show an overt neurological phenotype but may develop subclinical synaptic deficits. Detailed electrophysiological analyses showed subtle changes in presynaptic neurotransmitter release. Acetylcholine release at 40 Hz nerve stimulation at aged GM2synthase *null*-mutant NMJs ran down slightly more pronounced than at wildtype NMJs, and spontaneous acetylcholine release rate at GD3synthase *null*-mutant NMJs was somewhat higher than at wildtype, selectively at 25 °C bath temperature. Interestingly, we observed faster kinetics of postsynaptic electrophysiological responses at aged GD3synthase *null*-mutant NMJs, not previously seen by us at NMJs of young GD3synthase *null*-mutants or other types of (aged or young) ganglioside-deficient mice. These kinetic changes might reflect a change in postsynaptic acetylcholine receptor behaviour. Our data indicate that it is highly unlikely that transmission failure at NMJs contributes to the progressive motor defects of aged GM2synthase *null*-mutants and that, despite some kinetic changes of synaptic signals, neuromuscular transmission remains successful in aged GD3synthase *null*-mutant mice. Apparently, mutual redundancy of the different gangliosides in supporting presynaptic function, as observed previously by us in young mice, remains adequate upon ageing or, alternatively, gangliosides have only relatively little direct impact on neuromuscular synaptic function, even in aged mice.

Acknowledgements

This work was sponsored by a grant from the Prinses Beatrix Fonds (#MAR04-0213, to JJP) and from the Wellcome Trust (060349, 077041, to HJW). We thank Marloe Pijnacker for excellent caretaking of the mouse breedings.

Introduction

Gangliosides are sialic acid-containing glycosphingolipids (Ledeen, 1985) that are enriched in neuronal membranes and, therefore, are thought to have neuron-specific functions, e.g. in modulation of membrane-bound enzymes and ion-channels, cell-adhesion, neuritogenesis and membrane stability (Hakomori, 2003; Hashiramoto et al., 2006; Ledeen and Wu, 2006a; Salazar et al., 2004; Sohn et al., 2006; Susuki et al., 2007b; Wu et al., 2007; Yates and Rampersaud, 1998). Furthermore, anti-ganglioside autoimmunity and ganglioside turnover deficits cause neurological disorders (Ang et al., 2004; Maegawa et al., 2006; Simpson et al., 2004; Willison and Yuki, 2002).

Gangliosides may be particularly important in synaptic function because they 1) show high expression at synapses (Ando et al., 2004; Waki et al., 1994); 2) co-localize with neuroexocytotic proteins (Chamberlain et al., 2001; Lang et al., 2001; Salaün et al., 2004; Taverna et al., 2004) and 3) influence neurotransmitter release and synaptic plasticity when applied exogenously (Ando et al., 1998; Egorushkina et al., 1993; Furuse et al., 1998; Ramirez et al., 1990; Tanaka et al., 1997; Wieraszko and Seifert, 1985). In spite of this, we recently showed near-normal synapse function at neuromuscular junctions (NMJs) of mice lacking either complex gangliosides, b/c-series gangliosides or all gangliosides except GM3 (Figure 2.8) (Bullens et al., 2002; Zitman et al., 2008), suggesting neurotransmission was supported by GM3 alone or, alternatively, is ganglioside-independent.

One reason for this ganglioside redundancy could be the relatively young age (5-15 weeks) of the mice we investigated. Specific neuronal ganglioside levels vary during embryogenesis but also in postnatal phases, especially during ageing (Ando, 1983; Aydin et al., 2000; Malisan and Testi, 2002; Ngamukote et al., 2007; Svennerholm et al., 1989; Yamamoto et al., 2008). Gangliosides seem to become increasingly important upon ageing, as late-onset and progressive histological and behavioural abnormalities develop in both GM2synthase knockout (GM2s-KO) mice and compound *null*-mutant mice lacking GM2- and GD3synthase. Young GM2-KO mice (~3 months) show no phenotype (although they have histological signs of peripheral nerve degeneration (Sheikh et al., 1999), worsening upon ageing (Sugiura et al., 2005)). Eventually, starting at 8-16 months of age, these mice develop motor deficits, i.e. abnormalities in gait (Chiavegatto et al., 2000; Sugiura et al., 2005), balance, coordination, rearing and locomotion, whole-body tremor and catalepsy (Chiavegatto et al., 2000). The exact sequence of the development of these disturbances and the potential correlated neurodegenerative sites (i.e. sensory nerves, motor nerves or CNS) are not known yet. Spinal cord synaptic rearrangements occur in >10 weeks-old mice (Sugiura et al., 2005). Young GD3synthase knockout (GD3s-KO) mice have no phenotype (Kawai et al., 2001; Okada et al., 2002) but show impaired nerve regeneration (Okada et al., 2002). To our knowledge, they have not been studied at old age. Compound *null*-mutant mice are born normal but show sudden death (~50% survival at three (Kawai et al., 2001) or six months (Inoue et al., 2002), depending on strain) and get skin lesions at >6 months of age, presumably due to sensory nerve impairment (Inoue et al., 2002). Thus, these late-onset and progressive phenotypes may be due to a lack of the particular ganglioside sub-sets in these *null*-mutants. Alternatively, they might be caused by accumulation of the remaining ganglioside sub-sets, amounting to neurotoxic levels during ageing. Brains of GM2s-KO mice accumulate GM3 and GD3 ganglioside (Kawai et al., 2001; Takamiya et al., 1996), while those of GD3s-KO mice accumulate a-series gangliosides (Handa et al., 2005;

Okada et al., 2002) and possibly also O-series gangliosides. Brains of compound *null*-mutants have increased GM3 expression (Inoue et al., 2002; Kawai et al., 2001). Our previous studies on pathophysiological effects of anti-ganglioside antibodies suggest that similar ganglioside accumulations also take place at NMJ motor nerve terminals of these mutants (Bullens et al., 2002; Goodfellow et al., 2005). The exact time course of these ganglioside accumulations in brain and NMJ are not known.

In view of these progressive behavioural and histological deficits, possibly involving progressive synaptic dysfunction due to either chronic lack of ganglioside subsets or toxic overexpression of the remaining subsets, we here investigated neurotransmission at NMJs of aged (i.e. >9 months-old) GM2s-KO and GD3s-KO mice.

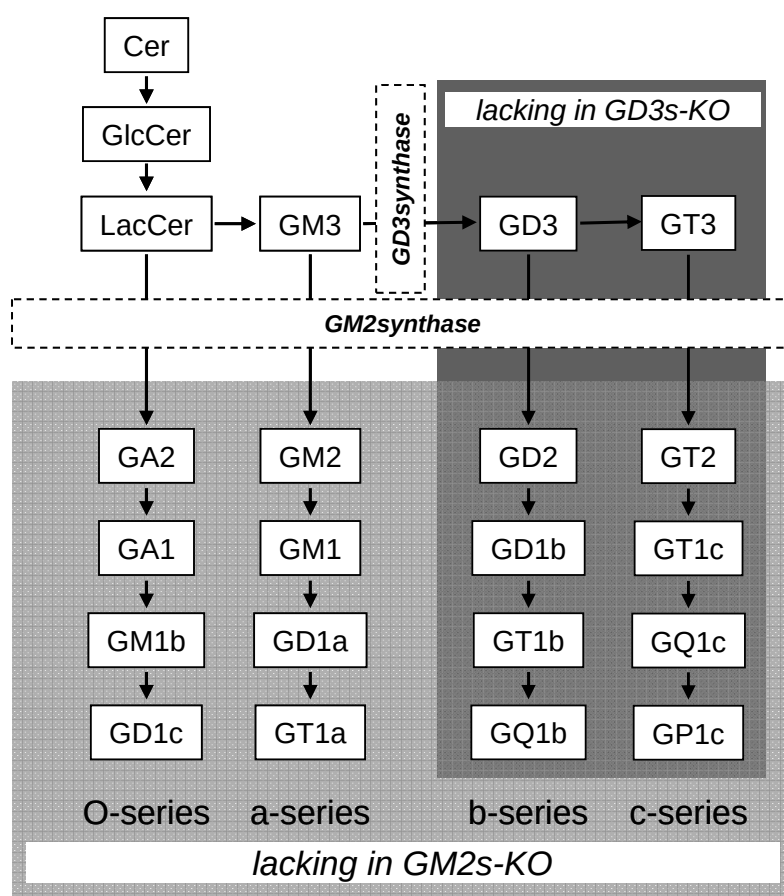


Figure 2.8 Gangliosides synthesis scheme

Ganglioside nomenclature is according to Svennerholm (Svennerholm, 1994). Membranes of wildtype mice contain all gangliosides. GM2s-KO mice lack complex gangliosides (light gray rectangle); GD3s-KO mice lack b- and c-series gangliosides (dark grey rectangle).

Material and methods

Mice

GM2s-KO and GD3s-KO mice were bred and genotyped as described by Inoue and colleagues (2002). Both male and female homozygous KO and wildtype (WT) littermates were used at ages older than 9 months (range 9.5-17 months). Upon visual inspection, aged homozygous GM2s-KO mice showed impaired motor behaviour (see

Introduction section), as described (Chiavegatto et al., 2000; Sugiura et al., 2005). The aged GD3s-KO mice showed no overt neurological phenotype. The body weight of the aged GD3s-KO mice was higher than that of the aged WT and GM2s-KO mice (47.1 ± 1.8 , 41.5 ± 1.5 and 36.6 ± 1.4 g, respectively; $p < 0.01$). Muscle strength was assessed with a grip strength meter as described before (Zitman et al., 2008). All animal experiments were carried out according to Dutch law and Leiden University guidelines.

***In vitro* electrophysiology**

Mice were killed by CO₂ asphyxiation. Left and right hemi-diaphragms were dissected with their phrenic nerve attached and mounted in standard Ringer's medium (119 mM NaCl, 4.5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM NaH₂PO₄, 23 mM NaHCO₃, 11 mM glucose, pH 7.4) at room temperature, pre-gassed with 95% O₂ / 5% CO₂.

Intracellular recordings of miniature endplate potentials (MEPPs) and endplate potentials (EPPs) in the NMJ were made using a glass micro-electrode (10-20 MΩ, filled with 3 M KCl) connected to a Geneclamp 500B (Axon Instruments / Molecular Devices) for amplifying and filtering (10 kHz low-pass, 1 Hz high-pass) of the signal. Signals were digitized and analyzed using a Digidata 1322A digitizer, Clampex 9, Clampfit 9 (Axon instruments) and Mini Analysis 6.0.3 (Synaptosoft). At least 10 NMJs were sampled per muscle per experimental condition and at least 30 EPPs and/or 40 MEPPs were sampled per NMJ.

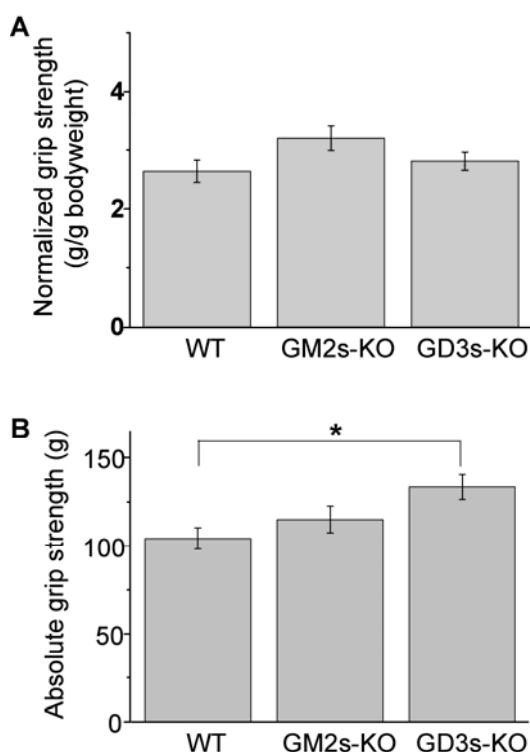


Figure 2.9. Grip strength measurements

Grip strength was averaged for 10 trials ($n=6-8$ mice). **A.** Pulling force normalized to the body weights of each mouse; all groups pulled ~3 g/g bodyweight.

B. Absolute pulling values show increased grip strength in GD3s-KO vs. WT mice ($p < 0.05$), paralleling their higher body weight.

Muscle action potentials were eliminated by incubation with 3 μM of the selective skeletal muscle Na⁺-channel blocker, μ-conotoxin GIIIB (Scientific Marketing Associates). For recording of EPPs, the phrenic nerve was stimulated with

supramaximal rectangular pulses of 0.1 ms width at 0.3, 3 and 40 Hz, using a bipolar stimulation electrode. The mean EPP and MEPP amplitudes at each NMJ were normalized to a standard resting membrane -75 mV, with the reversal potential for acetylcholine (ACh)-induced current assumed to be 0 mV (Magleby and Stevens, 1972) and using the following formula: $(M)EPP_{\text{normalized}} = (M)EPP \times ((-75 - V_{\text{reversal}})/V_m)$, where V_{reversal} is the reversal potential for ACh and V_m is the measured resting membrane potential. In order to calculate the quantal content (i.e. the number of ACh quanta that are released upon a single nerve impulse), the normalized mean amplitude of EPPs evoked at low rate (0.3 Hz) were corrected for non-linear summation using the following formula: $EPP_{\text{corrected}} = EPP/(1-f(EPP/75))$, with f set to 0.8 (McLachlan and Martin, 1980). The normalized and corrected mean EPP at each NMJ amplitude was divided by the normalized mean MEPP amplitude to obtain the quantal content value. Temperature of the bath medium was controlled using a Peltier device placed around the recording bath and adjusted by varying the DC output of a power supply (Delta Elektronika). A miniature probe connected to a digital thermometer was used to monitor temperature, which was held at 24-26 °C, unless stated otherwise.

MEPPs were also recorded after addition of hypertonic medium (0.5 M sucrose Ringer's medium), in order to estimate the pool of ACh vesicles ready for immediate release (Stevens and Tsujimoto, 1995; Varoqueaux et al., 2005). In some experiments we varied the concentrations of extracellular K^+ and Ca^{2+} by adding the appropriate amounts of KCl and $CaCl_2$, respectively, to the Ringer's medium.

Statistical analysis

Data are presented as group mean $\pm$ SEM of the mean muscle values calculated from the mean NMJ values. Statistical differences were analyzed with an unpaired Student's t -test or an analysis of variance (ANOVA) with Bonferroni's post-hoc test.

Results

In vivo assessment of muscle strength

Grip strength testing showed similar muscle power of WT, GM2s-KO and GD3s-KO mice, when normalized for body weight (~ 3 g/g; $p = 0.13$; Figure 2.9A). The absolute force delivered by GD3s-KO mice was $\sim 20\%$ higher than that delivered by WT mice ($p < 0.05$; Figure 2.9B), paralleling the higher body weight of this mutant.

Spontaneous and evoked ACh release parameters

First we measured synaptic signals at NMJs under our standard conditions (2 mM extracellular Ca^{2+} , 25 °C). Each genotype group consisted of 6-11 mice. Spontaneous unquantal transmitter release, measured as MEPP frequency, was 43% larger in GD3s-KO than in WT NMJs (1.91 ± 0.23 and 1.34 ± 0.09 s $^{-1}$, respectively, $p < 0.05$; Figure 2.10A). GM2s-KO MEPP frequency was 1.42 ± 0.11 s $^{-1}$ and did not differ statistically significantly from the other genotype groups. MEPP amplitude in the GD3s-KO NMJ was $\sim 25\%$ larger than in WT or GM2s-KO (0.79 ± 0.05 ; 0.63 ± 0.03 ; 0.65 ± 0.02 mV, respectively; $p < 0.05$; Figure 2.10B, C). In addition, we found a $\sim 30\%$ shorter 10-90% rise time of MEPPs in the GD3s-KO mice, as compared to GM2s-KO and WT (0.72 ± 0.04 , 1.01 ± 0.07 and 1.06 ± 0.05 ms, respectively,

$p < 0.01$) as well as a ~25% smaller halfwidth (2.29 ± 0.08 , 2.92 ± 0.23 and 3.06 ± 0.15 ms, respectively, $p < 0.05$). The amplitudes of EPPs evoked by 0.3 Hz stimulation were 22-24 mV for all groups ($p = 0.22$; Figure 2.10D, E). The calculated quantal contents of 0.3 Hz EPPs were of similar value (~48) at all three genotype NMJs ($p = 0.23$; Figure 2.10F). However, the 10-90% rise time of GD3s-KO EPPs was shorter than that of WT and GM2s-KO (0.81 ± 0.05 , 1.13 ± 0.07 and 1.11 ± 0.05 ms, respectively; $p < 0.01$; Figure 2.10G). Similarly, the width of the EPPs at 50% of their amplitude was smaller in GD3s-KO than WT, but not GM2s-KO NMJs (2.93 ± 0.11 , 3.89 ± 0.14 and 3.49 ± 0.28 ms, respectively; $p < 0.05$ GD3s-KO vs. WT; Figure 2.10H). Example traces are shown in Figure 2.10E. These kinetic differences were not observed previously in NMJs of younger GD3s-KO mice (Zitman et al., 2008). Upon high rate (40 Hz) stimulation, GM2s-KO EPPs ran down to a slightly, but statistically significantly ($p < 0.05$), lower plateau level than the WT EPPs did (79.5 ± 1.0 and $83.2 \pm 0.9\%$ of the first EPP in the train, respectively; Figure 2.11). GD3s-KO EPPs ran down to $82.1 \pm 0.8\%$. Within the tested groups of old GM2s- and GD3s-KO mice we observed no clear correlation between the age of individual mice (which ranged from 9.5 to 17 months) and the parameters that had shown statistically significant change compared with the WT group on group-average level (data not shown).

Hypertonic shock- and K^+ -induced ACh release

Upon incubation of nerve-muscle preparations in hypertonic (0.5 M sucrose) Ringer's medium, MEPP frequency increased to similar levels of $50\text{--}60\text{ s}^{-1}$ for all groups ($n = 6$ mice; $p = 0.16$; Figure 2.12A). Cultured GM2s-KO neurons have been shown to degenerate upon K^+ -induced depolarization, caused by an underlying impairment of cellular Ca^{2+} regulation (Wu et al., 2001). In order to investigate possible effects at the GM2s-KO NMJ, we measured MEPP frequency in extracellular K^+ concentrations varying from 4.5 to 30 mM and compared it to WT NMJs ($n = 3\text{--}7$ mice per condition per genotype). As expected, the MEPP frequency steeply rose with increasing K^+ concentration, but we found no statistically significant differences between the genotypes (Figure 2.12B).

Temperature-dependency of ACh release parameters

Gangliosides may be involved in thermal adaptation of neuronal membrane function (Rahmann et al., 1998). We, therefore, performed synaptic electrophysiological measurements in a further series of experiments at 17, 20, 30, and 35 °C (Figure 2.13). Each aged genotype group consisted of 4-5 mice. At all temperatures tested, the MEPP amplitude at GD3s-KO NMJs tended to be somewhat higher at GD3s-KO NMJs than at GM2s-KO and WT NMJs, although this was only statistically significantly at 25 °C, as described above (Figure 2.13A; $p < 0.01$). The temperature-dependence of spontaneous ACh release rate, i.e. MEPP frequency, seemed roughly similar at WT, GM2s-KO and GD3s-KO NMJs, although at the low end of the tested temperature range the MEPP frequency at WT NMJs was somewhat higher, compared to GM2s-KO (Figure 2.13B; $p < 0.05$). The amplitudes of EPPs evoked by 0.3 Hz nerve stimulation amplitudes tended to be somewhat higher at GD3s-KO NMJs at every temperature tested, although this reached no statistical significance (Figure 2.13C). No differences in the calculated quantal contents were observed at any temperature tested (Figure 2.13D). Rise times and halfwidths of GD3s-KO EPPs were consistently shorter than that of WT and GM2s-KO EPPs, but showed no different temperature-dependence profile (Figure 2.13E, F).

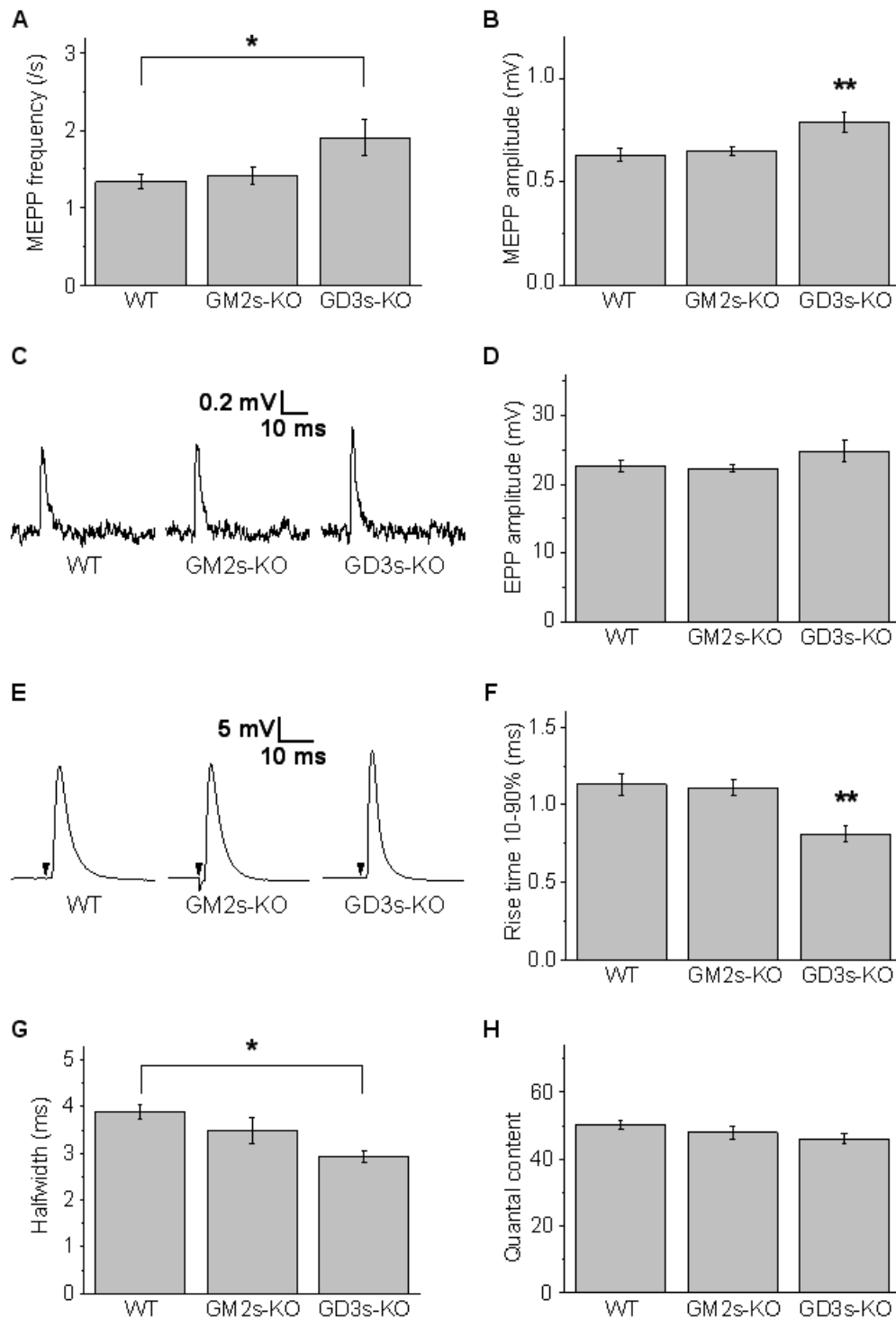


Figure 2.10. Basic synaptic parameters at the NMJ

In vitro intracellular electrophysiological measurements at 25 °C in diaphragm-phrenic nerve preparations: each group consisted of at least 6 mice; at least 10 NMJs were sampled per muscle.

A. MEPP frequency was higher in the GD3s-KO NMJs compared to WT. **B.** MEPP amplitude was larger at GD3s-KO NMJs. **C.** Typical traces of MEPPs. **D.** EPP amplitude was similar at each genotype. **E.** EPP example traces for WT, GM2s-KO and GD3s-KO mice. Black triangles indicate moment of phrenic nerve stimulation. **F.** Calculated quantal content at 0.3 Hz nerve stimulation was similar across genotypes. **G.** GD3s-KO mice exhibit significantly shorter 10-90% rise time. **H.** EPP halfwidth at GD3s-KO NMJs was shorter than at WT.

*p<0.05, **p<0.01

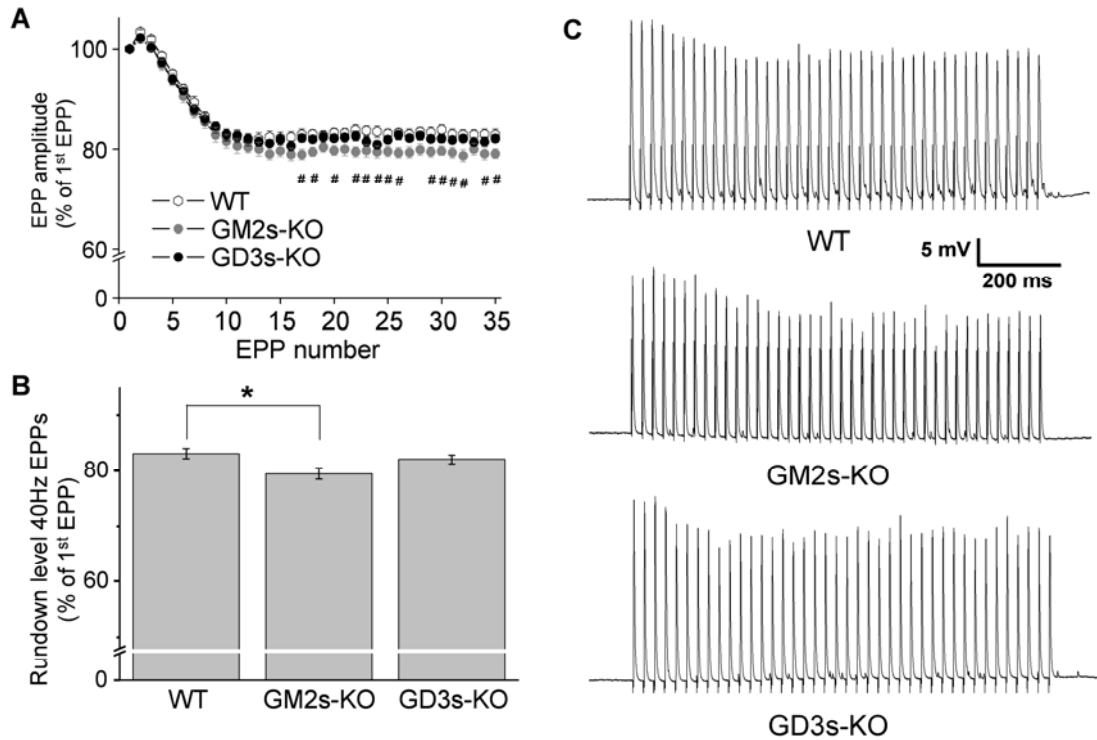


Figure 2.11. High-rate nerve stimulation-evoked ACh release is reduced at GM2s-KO NMJs

A. EPP amplitude rundown profiles at 40 Hz nerve stimulation ($n=6-11$ mice per group). Each EPP amplitude is expressed as percentage of the amplitude of the first EPP. EPPs at GM2s-KO NMJs showed increased rundown, compared to WT (# $p<0.05$). **B.** EPP rundown level (the mean amplitude of the 21st-35th EPP, expressed as percentage of the amplitude of the first EPP in the train), was lower at GM2s-KO NMJs, compared to WT (* $p<0.05$). **C.** Typical 40 Hz nerve stimulation EPP trains.

No differences were observed between genotypes in rundown of EPPs at 3 Hz stimulation rate (Figure 2.13G), while at 40 Hz the WT EPPs ran down slightly less pronounced than at ganglioside-deficient NMJs, albeit only statistically significantly at 25 °C ($p<0.05$, vs. GM2s-KO) and 35 °C ($p<0.05$, vs. both GD3s-KO and GM2s-KO; Figure 2.13H).

Ca²⁺-dependency of ACh release parameters

Gangliosides have been suggested to play a role in the membrane flux of Ca²⁺ and its cellular homeostasis (Ledeen and Wu, 2006a; Rahmann et al., 1998; Wu et al., 2001). Therefore, we explored synaptic transmission at NMJs from muscle preparations incubated at low (0.2 mM) and high (5 mM) extracellular Ca²⁺, at 25 °C bath temperature. Genotypes showed no differences in MEPP amplitude at either 0.2 or 5 mM Ca²⁺ concentration (Figure 2.14), whereas at 2 mM Ca²⁺ it was somewhat higher at the GD3s-KO NMJ (see above). As expected, in view of the well-known dependence of neurotransmitter release on extracellular Ca²⁺, MEPP frequency, EPP amplitude and quantal content of WT NMJs steeply depended on Ca²⁺. No different Ca²⁺-dependency of these parameters was observed at GM2s-KO NMJs. However, the dependency of GD3s-KO NMJs seemed less steep, as we found increased ACh release at 0.2 mM Ca²⁺, compared to WT and GM2s-KO NMJs (Figure 2.14B-D). EPP rundown levels at 40 Hz stimulation were not statistically significantly different between genotypes at 0.2 and 5 mM Ca²⁺ (Figure 2.14E); whereas they differed

somewhat at 2 mM Ca^{2+} , see above. Paired-pulse facilitation at 25 ms stimulation interval was not different between genotypes at any of the Ca^{2+} concentrations tested. Its values, expressed as percentage increase of the second EPP, compared to the first, were about 25, 2.5 and -0.5% at 0.2, 2, and 5 mM Ca^{2+} , respectively, for all genotypes. As observed at 2 mM Ca^{2+} (see above), the rise time and halfwidth of EPPs were shorter at GD3s-KO than at WT and GM2s-KO NMJs at both 0.2 and 5 mM Ca^{2+} (although only statistically significantly at 5 mM Ca^{2+} , Figure 2.14F, G). The extent of this difference, however, was similar at all Ca^{2+} concentrations.

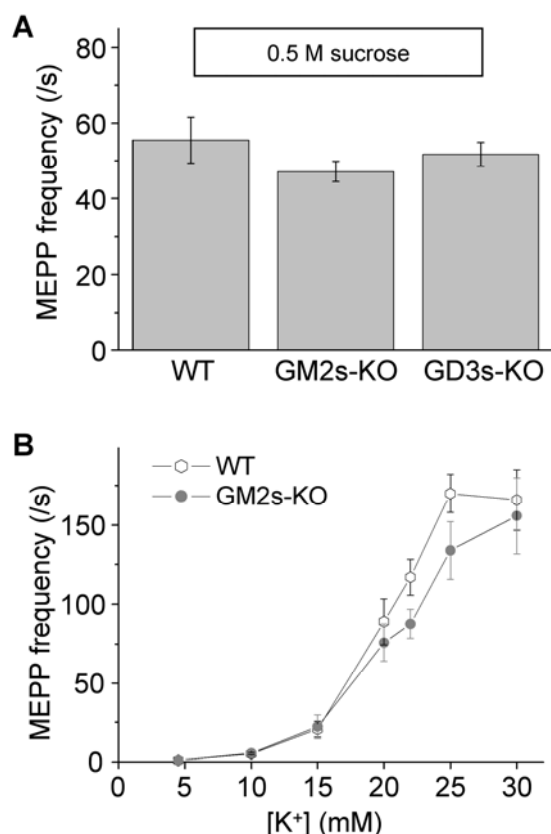


Figure 2.12 Hypertonic shock- and K⁺-induced ACh release is unchanged

A. The levels of ACh release induced by hypertonic medium (0.5 mM sucrose Ringer's medium) were not different between genotypes ($n=6$ mice).

B. No differences in K⁺-MEPP frequency concentration-effect relationship between genotypes ($n=3-7$ mice).

Discussion

We performed a detailed electrophysiological characterization of ACh release parameters at NMJs of aged GM2s-KO and GD3s-KO mice. This included high- and low-rate nerve stimulation-evoked release, spontaneous release and the release evoked by hypertonic medium or elevated extracellular K⁺. However, neuromuscular synaptic transmission of both the aged *null*-mutant mice showed remarkably little change compared with the WT controls. From the evoked release parameters under standard conditions (25 °C, 2 mM extracellular Ca^{2+}), only the ACh release at 40 Hz nerve stimulation at aged GM2s-KO NMJs was found to be running down more pronouncedly than at WT NMJs. However, the extent of this difference was only small (EPPs ran down to a plateau level of 79.5% of the first EPP, compared to 83.2% at WT NMJs). In view of the presence of a large safety factor in neuromuscular transmission (Wood and Slater, 2001), such a small reduction will most likely not threaten successful transmission and cause muscle weakness. This was confirmed in

testing the aged GM2s-KO mice with a grip strength meter, where they pulled equal forces as WT controls. Thus, the motor coordination defects observed in aged GM2s-KO mice (Chiavegatto et al., 2000; Sugiura et al., 2005), and also observed by us in the mice used here, do not contain a muscle weakness component due to neuromuscular transmission failure. Therefore, they are likely attributable to progressive dysfunction of the peripheral sensory or proximal motor nerves and/or possibly the CNS, caused by chronic lack of complex gangliosides or, alternatively, by upregulation of GM3 and GD3 ganglioside to toxic levels. Neurotransmission at central synapses has no safety factor and, consequently, small changes in transmitter release as observed here at the NMJ already may have pronounced effects on neuronal network function in the CNS.

Earlier we have also observed some extra rundown of high-rate EPPs at NMJs of young GM2s-KO mice, but only at higher bath temperatures (30-35°C) tested and at high (5 mM) extracellular Ca^{2+} concentration (Bullens et al., 2002). Furthermore, GM2*GD3synthase compound *null*-mutant NMJs showed some degree of extra EPP rundown, at all temperature and Ca^{2+} conditions tested (Zitman et al., 2008). However, at GD3s-KO NMJs, either from young or aged mice, we never observed extra EPP rundown compared with WT, except for the 35 °C temperature condition at aged NMJs. Together these data suggest that O- and/or a-series gangliosides may support the fine-tuning of high-rate transmitter release. In particular, GM1 ganglioside may be important, because it has been shown to influence cellular Ca^{2+} membrane flux and homeostasis (Wu et al., 2004; Wu et al., 1990). Perhaps this involves inhibition of voltage-gated Ca^{2+} -channel inactivation, one important factor in high-rate transmitter release. Another relevant factor, the size of the pool of synaptic vesicles ready for release, is not likely to be influenced by gangliosides, because we did not find differences between genotypes when we assessed for this parameter with hypertonic stimulation (Stevens and Tsujimoto, 1995; Varoqueaux et al., 2005), either here at aged NMJs or previously in younger ones (Zitman et al., 2008). The less steep relationship between extracellular Ca^{2+} and evoked ACh release observed at NMJs of old GD3s-KO mice (and seen previously in young ones) may also be due to altered Ca^{2+} buffering or membrane flux, possibly resulting from a progressive increase in GM1 density due to accumulation of O- and/or a-series gangliosides (Handa et al., 2005; Okada et al., 2002). This may also explain the higher MEPP frequency at GD3s-KO NMJs in 0.2 and 2 mM extracellular Ca^{2+} .

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-KO or any of the other ganglioside-deficient mice. These kinetic changes may reflect a change in the behaviour of postsynaptic ACh receptors (AChRs). Gangliosides are present in the vicinity of AChRs (Pato et al., 2008) and can interact with the protein, as shown for AChR-rich membrane from *Torpedo marmorata* (Mantipragada et al., 2003). Accumulation of O- and/or a-series gangliosides may occur at muscle membranes of GD3s-KO mice, as it does at neuronal membranes (Handa et al., 2005; Okada et al., 2002), and progressiveness of this process may lead to the observed age-specific changes in EPP kinetics. EPP rise time and halfwidth are highly temperature-dependent parameters (Figure 2.13E, F) and the progressive accumulation of O- and/or a-series gangliosides may induce a shift in this dependency by progressively altering the membrane fluidity and charge density in the vicinity of the AChRs in a similar way that gangliosides have been shown to influence artificial voltage-gated ion-channels in lipid bilayers in a temperature-dependent fashion (Kappel et al., 2000).

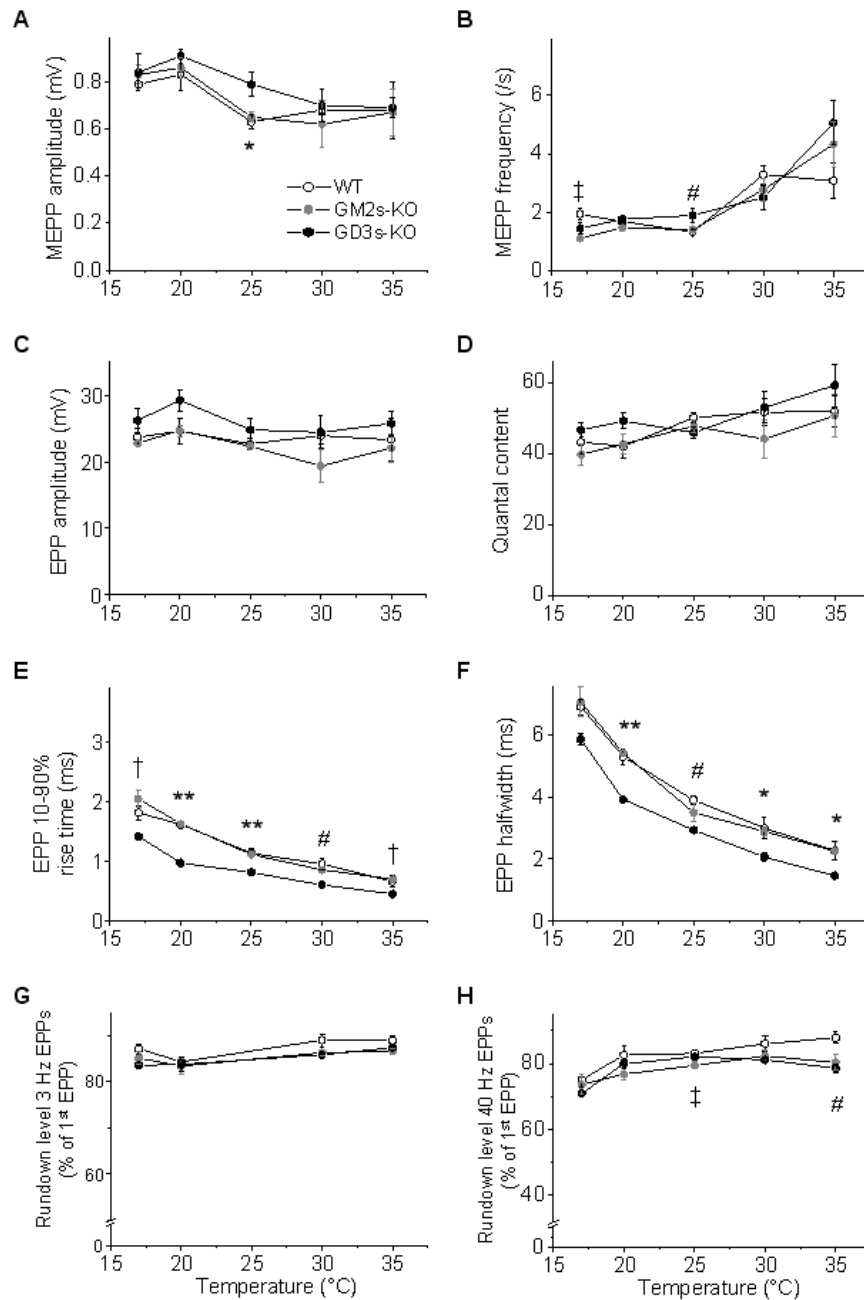


Figure 2.13. Effect of temperature on synaptic transmission

Electrophysiological analysis of ACh release at diaphragm NMJs at different bath temperatures. Each genotype group consisted of 4-5 mice. **A.** MEPP amplitude at GD3s-KO mice was larger than at GM2s-KO and WT NMJs, only at 25 °C. **B.** Spontaneous ACh release, measured as MEPP frequency, was lower at GM2s-KO than WT NMJs at 17 °C ($p < 0.05$), and higher at GD3s-KO compared with WT NMJs at 25 °C. **C.** No differences in temperature-dependency between genotypes of EPP amplitude at 0.3 Hz nerve stimulation. **D.** The temperature-dependency of quantal content at 0.3 Hz nerve stimulation was similar across genotypes. **E.** GD3s-KO NMJs showed equal reductions of rise time at all temperatures tested. **F.** Halfwidths of EPPs at GD3s-KO NMJs were shorter than at WT and GM2s-KO NMJs at temperatures > 20 °C. **G.** No changes in temperature-dependency of EPP amplitude rundown level at 3 Hz stimulation. **H.** EPP amplitude rundown level at 40Hz nerve stimulation. GM2s-KO rundown was somewhat more pronounced than WT at 25 °C and GD3s-KO rundown was more pronounced than WT at 35 °C.

* $p < 0.05$, ** $p < 0.01$, WT vs. GM2s-KO and GD3s-KO; † $p < 0.05$, GD3s-KO vs. GM2s-KO; # $p < 0.05$, GD3s-KO vs. WT; ‡ $p < 0.05$, GM2s-KO vs. WT.

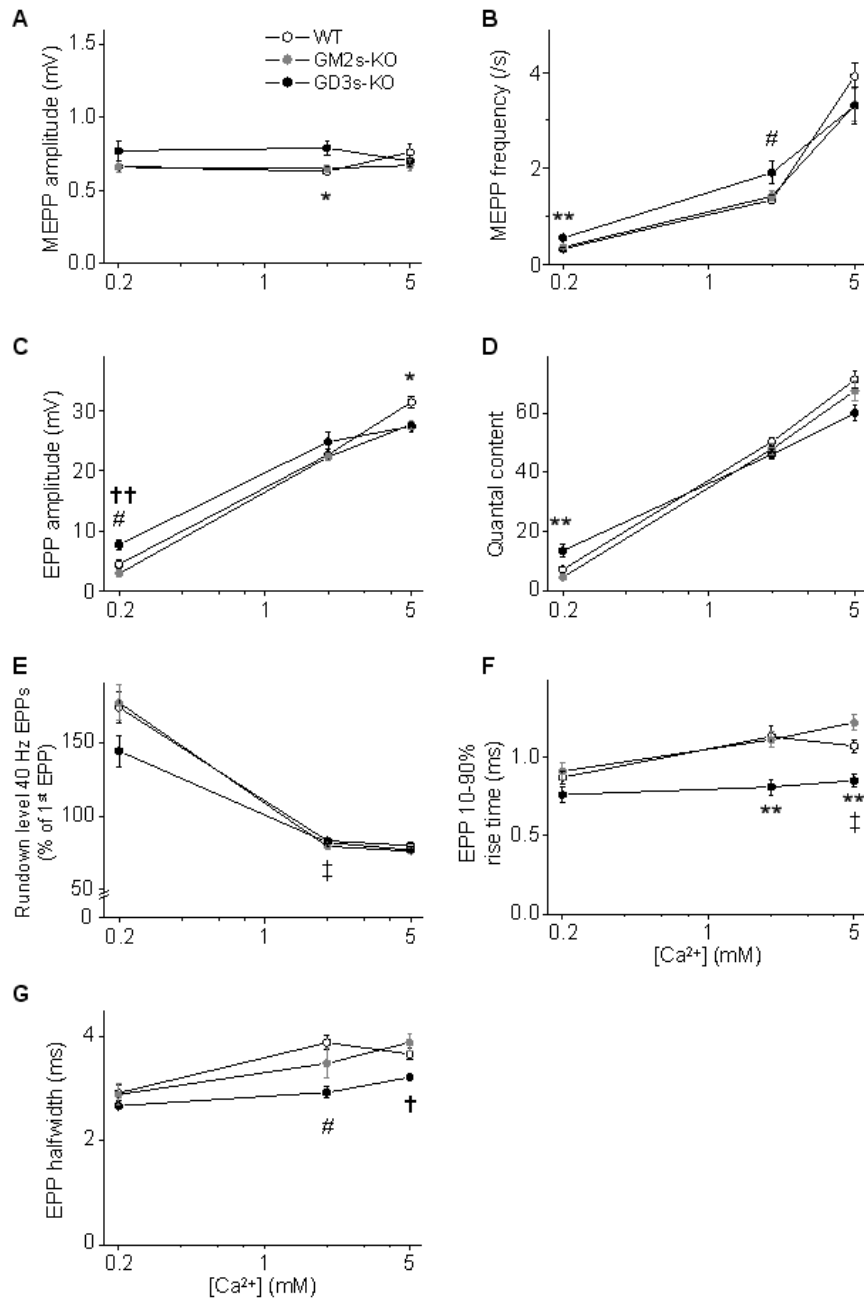


Figure 2.14. Effect of extracellular Ca^{2+} concentration on synaptic transmission

Electrophysiological measurements were performed in Ringer's medium at 25 °C containing either 0.2, 2 or 5 mM Ca^{2+} ; $n=6-11$ mice per group. **A.** MEPP amplitude at GD3s-KO NMJs was larger than at GD3s-KO and WT NMJs at 2 mM Ca^{2+} . **B.** Spontaneous ACh release, measured as MEPP frequency, was higher at GD3s-KO NMJs compared with the other genotypes at 0.2 mM Ca^{2+} and higher than WT at 2 mM Ca^{2+} . **C.** EPP amplitude was increased at GD3s-KO NMJs at low Ca^{2+} conditions and was lower than WT in high Ca^{2+} . **D.** Quantal content at 0.3 Hz nerve stimulation in 0.2 mM Ca^{2+} was higher at GD3s-KO NMJs. **E.** EPP rundown level was only somewhat lower for GM2s-KO in 2 mM Ca^{2+} . **F.** GD3s-KO mice exhibit smaller EPP rise times at 2 and 5 mM Ca^{2+} . **G.** Similarly, halfwidths of GD3s-KO EPPs were reduced

* $p<0.05$, ** $p<0.01$, WT vs. GM2s-KO and GD3s-KO; $\dagger$ $p<0.05$, $\dagger\dagger$ $p<0.01$, GD3s-KO vs. GM2s-KO; $\#$ $p<0.05$, GD3s-KO vs. WT; $\ddagger$ $p<0.05$, GM2s-KO vs. WT.

The small increase in MEPP amplitude at aged GD3s-KO, specifically at 25 °C and not observed at young GD3s-KO NMJs, may possibly have followed from an influence by gangliosides on ion permeation through AChRs (Barrantes, 1989), progressively changing due to O- and/or a-series gangliosides accumulation. Alternatively, the increased MEPP amplitude may be due to reduced activity of cholinesterase in the synaptic cleft, inhibiting ACh breakdown. However, in that case one would expect an accompanying MEPP and EPP broadening (due to a prolonged action of ACh, as shown in many electrophysiological experiments using cholinesterase inhibitors, see e.g. Fiekers, 1985; Maselli and Leung, 1993), but this was not found here. Instead, we observed faster MEPPs and EPPs. These might have resulted from increased cholinesterase activity, but this would in turn be expected to be associated with reduced amplitude, which was not the case. Furthermore, to our knowledge, no direct relationship between gangliosides and cholinesterase has been reported in the literature. These considerations make changes in cholinesterase activity a more distant possibility. A further alternative explanation for changes of (M)EPP kinetics may be that O- and/or a-series accumulation influenced the passive electrical characteristics of muscle membrane (i.e. reduced capacitance and increased input resistance). A presynaptic explanation for the changed EPP and MEPP kinetics and quantal size (e.g. increased filling of ACh vesicles) is less likely because gangliosides have been reported to lack effect on ACh synthesis (Tanaka et al., 1997). On the basis of the only relatively small magnitude of the observed kinetic changes of the EPPs and MEPPs in aged GD3s-KO mice, and the EPP amplitude being somewhat increased compared with WT, it is not to be expected that successful neuromuscular transmission is endangered. Absence of muscle weakness in grip strength measurements and the observed absence of any overt neurological symptoms in the aged GD3s-KO mice are in agreement with this view.

GM2s-KO cerebellar neurons have been shown to degenerate when cultured under K^+ -induced depolarization, caused by an underlying impairment of cellular Ca^{2+} regulation (Wu et al., 2001). We tested in acute experiments whether a similar effect occurs at aged GM2s-KO motor nerve terminals by measuring the increase in MEPP frequency evoked by increased extracellular K^+ concentrations. However, no differences were observed between GM2s-KO and WT NMJs, indicating no major differences between aged GM2s-KO and WT motor nerve terminals in influx or intracellular buffering of Ca^{2+} .

In conclusion, the investigated aged ganglioside-deficient mice show remarkable little change in presynaptic neuromuscular function, compared with age-matched WT controls. Apparently, the mutual redundancy of the different gangliosides in supporting synaptic function, as observed in young mice (Zitman et al., 2008), remains adequate even upon ageing, when there is likely an increased demand for (gangliosides-dependent) maintenance and repair processes. Alternatively, gangliosides may have relatively little direct impact on neuromuscular synaptic function, even at old age. The observation of fast EPP and MEPP kinetics at GD3s-KO NMJs is interesting and indicates a role for O- and/or a-series gangliosides in AChR function, which deserves more detailed study. Finally, in view of the only slightly reduced 40 Hz nerve stimulation-evoked ACh release at NMJs of aged GM2s-KO mice, it is highly unlikely that NMJ dysfunction contributes to the age-related motor defects of GM2s-KO mice.

CHAPTER 2.3

Total ganglioside ablation at mouse motor nerve terminals alters neurotransmitter release level

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Published in Synapse 64 (2010) 335 - 338

Abstract

Neuronal membrane gangliosides, forming a large family of sialylated glycosphingolipids, have been hypothesized to play important roles in synaptic transmission. We studied the *ex vivo* electrophysiological function of neuromuscular junctions of GM2/GD2synthase*GD3synthase compound *null*-mutant mice after acute removal of GM3, the only remaining ganglioside in this mouse, by *in vitro* treatment with neuraminidase. We found 16% enhancement of the acetylcholine release per nerve impulse at low-rate (0.3 Hz) nerve stimulation. Conversely, the treatment reduced the acetylcholine release evoked by high-rate (40 Hz) nerve stimulation. Also, 25 ms paired-pulse facilitation of endplate potentials was reduced by the neuraminidase-treatment. These effects may indicate a modest modulatory influence of the negative electrical charges carried by the sialic acid molecules of gangliosides on the function of presynaptic Ca_v2.1 channels, affecting the magnitude and kinetics of the Ca²⁺ influx that induces neurotransmitter release from the motor nerve terminal. Our results show that gangliosides are to some extent involved in neurotransmission at the neuromuscular junction, but that their presence is not an absolute requirement in this process.

Acknowledgements

This work was sponsored by a grant from the Prinses Beatrix Fonds (#MAR04-0213, to JJP) and from the Wellcome Trust (060349, 077041, to HJW). We thank Marloe Pijnacker and Robert van Leeuwen for excellent caretaking of the mouse breedings.

Gangliosides are glycosphingolipids carrying one or more sialic acid residues. This family of amphipathic molecules resides in the outer layer of cell membranes and is especially enriched in presynaptic nerve endings (Schwarz and Futerman, 1996; for review, see Plomp and Willison, 2009). Hence, gangliosides have been hypothesized to play important roles in synaptic transmission, possibly through affecting presynaptic voltage-gated ion-channels, Ca^{2+} homeostasis and/or surface charge. However, at the neuromuscular junction (NMJ) of transgenic mice lacking major subsets of gangliosides (Figure 2.15), we have previously shown that presence of the *complete* family of gangliosides is not strictly required for functional synaptic transmission. Electrophysiological measurement of acetylcholine (ACh) release at NMJs of GM2synthase knockout (GM2s-KO), GD3synthase knockout (GD3s-KO), or compound *null*-mutant mice lacking both GM2- and GD3synthase (dKO), showed only subtle changes and no failure of synaptic transmission was observed (Bullens et al., 2002; Zitman et al., 2008; Zitman et al., 2009). This suggests a fine-tuning role for gangliosides in transmitter release, rather than their presence being an absolute requirement. Alternatively, the different types of gangliosides might act mutually compensatory in supporting transmitter release. Such a compensatory action of GM3 ganglioside might explain the redundancy we found for the other gangliosides, because in all three investigated transgenic strains at least this ganglioside remains present in the neural membrane (Figure 2.15), most likely at highly accumulated level (Inoue et al., 2002). We here acutely removed the sialic acid component of this only remaining ganglioside at dKO NMJs, thereby creating a ganglioside-free nerve terminal, and studied the effect on transmitter release.

Male and female dKO mice (Inoue et al., 2002; Zitman et al., 2008) were used in the experiments, at ~11 weeks of age and 22.9 ± 1.1 g body weight. Mice were killed by CO_2 asphyxiation. Left and right hemi-diaphragms with phrenic nerves were dissected and pinned out in Ringer's medium (119 mM NaCl, 4.5 mM KCl, 2 mM CaCl_2 , 1 mM MgSO_4 , 1mM NaH_2PO_4 , 23 mM NaHCO_3 , 11 mM glucose, pH 7.4), pre-gassed with 95% O_2 /5% CO_2 . Animal experiments were carried out according to Dutch law and Leiden University guidelines.

dKO phrenic nerve hemi-diaphragm preparations (6 left and 4 right hemi-diaphragms from $n=10$ dKO mice) were treated with 2 units neuraminidase type V from *Clostridium perfringens* (Sigma-Aldrich) in 1 ml Ringer's medium for 1 h at 32 °C. This disrupts GM3 by cleaving the $\alpha 2,3$ -link between the sialic acid and galactose molecule (Perillo et al., 1994). We used tissue from dKO mice because neuraminidase treatment of wildtype tissue will not induce complete ganglioside ablation due to the inability of the neuraminidase to desialylate GM1 (Perillo et al., 1994). The contralateral hemi-diaphragm from each mouse was incubated as a control in Ringer's medium only. Preparations were then rinsed in Ringer's medium for 10 min. Subsequently, μ -conotoxin-GIIIB (3 μM ; Scientific Marketing Associates) was added to block voltage-gated Na^+ channels on the muscle membrane in order to prevent muscle action potentials and contraction. ACh release at NMJs at room temperature (20-22 °C) was assessed using an intracellular electrophysiological method, as described before (Zitman et al., 2008), recording endplate potentials (EPPs, the postsynaptic responses resulting from nerve action potential-evoked ACh release following supramaximal stimulation of the phrenic nerve) and miniature EPPs (MEPPs, the responses resulting from spontaneous unquantal release). The quantal content (i.e. the number of ACh quanta released per nerve impulse) was calculated from EPP and MEPP amplitudes (Zitman et al., 2008). Ten NMJs were sampled per muscle per experimental condition and at least 30 EPPs and 40 MEPPs were sampled

per NMJ. Data are expressed as group mean $\pm$ SEM ($n=10$ mice) of the mean muscle values calculated from the mean NMJ values. Statistical significance of differences between neuraminidase-treated and control groups was analyzed with paired Student's *t*-tests.

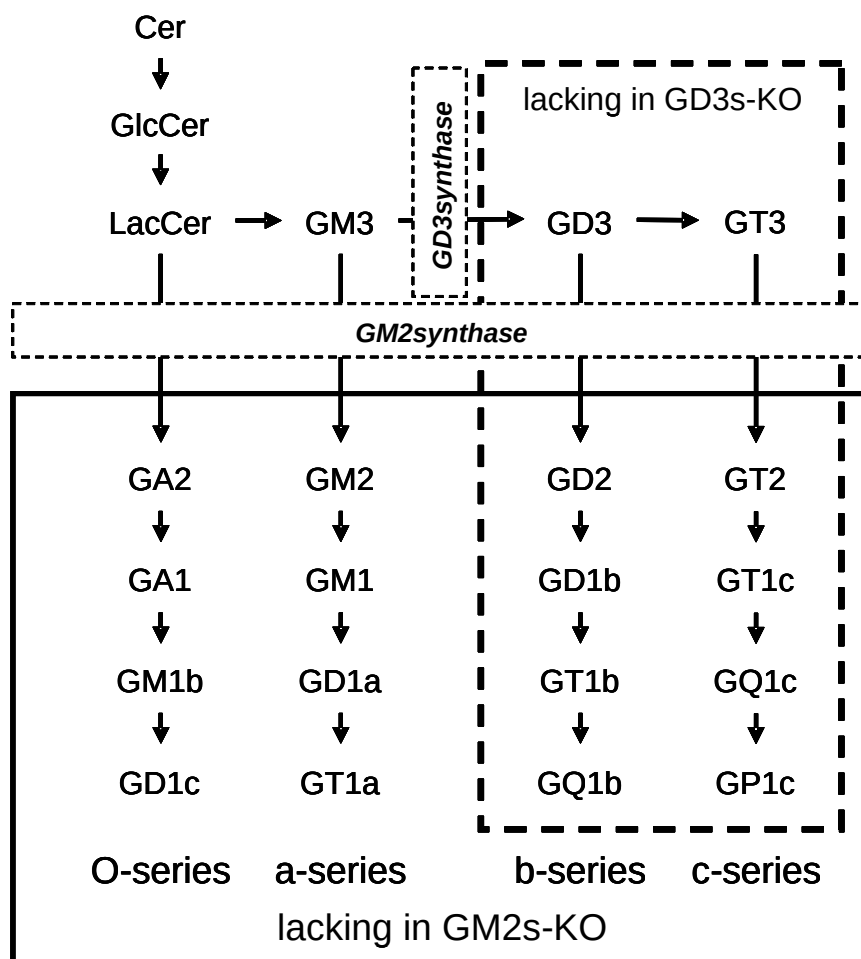


Figure 2.15. Ganglioside synthesis scheme with indication of the subsets lacking in GM2s-KO and GD3s-KO mice. dKO mice only express GM3.

The disruption of GM3 at the dKO NMJ by neuraminidase caused a 16% increase of quantal content at 0.3 Hz nerve stimulation (control 42.3 ± 2.1 and neuraminidase-treated 49.1 ± 1.6 ; $p < 0.05$; Figure 2.16A). Spontaneous unquantal ACh release, measured as MEPP frequency, showed a tendency of increase in the neuraminidase-treated NMJs, but this was not statistically significant ($p = 0.28$, Figure 2.16B). During high rate (40 Hz) nerve stimulation, ACh release at neuraminidase-treated dKO NMJs became more depressed than that at control dKO NMJs. The rundown level of EPPs (calculated as the mean amplitude of the 21st – 35th EPP, expressed as percentage of the first EPP of the train) at NMJs in neuraminidase-treated tissue was $67.3 \pm 1.5\%$, compared to $72.1 \pm 0.7\%$ at NMJs of untreated dKO muscles ($p < 0.05$; Figure 2.16C-E). For comparison, EPP rundown level at wildtype NMJs under comparable experimental conditions in our previous study was $80.9 \pm 1.2\%$ (Zitman et al., 2008). Furthermore, the increase of the second EPP of the 40 Hz evoked train, compared to the first EPP (i.e. 25 ms paired-pulse facilitation), was lower in the neuraminidase-treated group ($3.35 \pm 0.66\%$ at control vs. $0.32 \pm 0.50\%$ at neuraminidase-treated dKO NMJs; $p < 0.01$; Figure 2.16F).

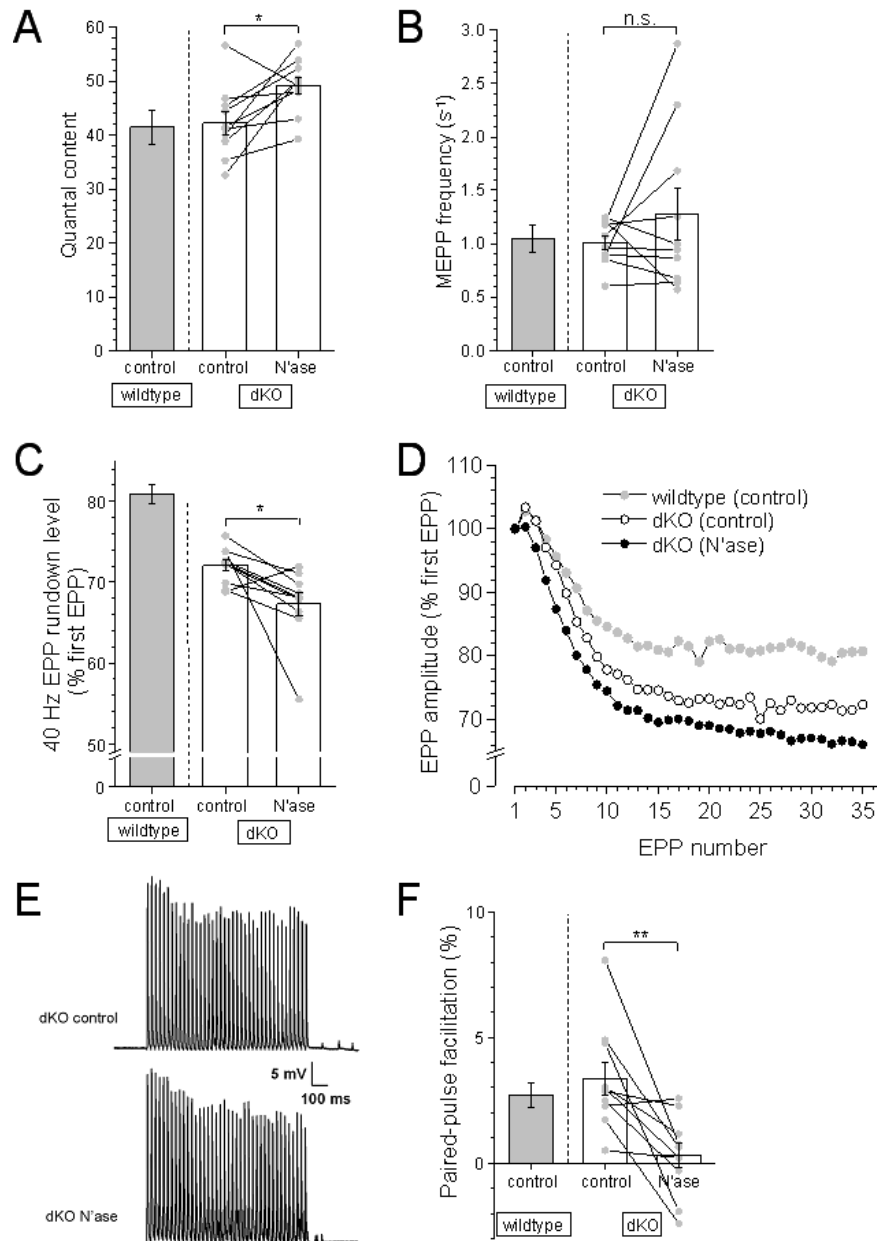


Figure 2.16 Electrophysiological measurement of ACh release at diaphragm NMJs of dKO mice ($n=10$) to assess the effect of neuraminidase (N'ase). Controls were sham-incubated without neuraminidase. For comparison, the values of synaptic parameters obtained at wildtype NMJ (without neuraminidase treatment) in our previous study (Zitman et al., 2008), have been added to the panels (gray bars, $n=4$ mice). Data are expressed as group means $\pm$ SEM (bars) and hemi-diaphragm pairs from individual mice (gray dots with connecting line). **A.** Quantal content at 0.3 Hz nerve stimulation increased 16% ($p<0.05$) by the neuraminidase treatment. **B.** MEPP frequency showed an increase, but this was not statistically significant ($p=0.28$). **C.** Rundown level of EPPs upon 40 Hz nerve stimulation was more pronounced ($p<0.05$). **D.** Mean EPP amplitude profiles during 40 Hz nerve stimulation. **E.** Representative examples of 40 Hz EPPs at control and neuraminidase-treated NMJs. **F.** Paired-pulse (25 ms) facilitation became smaller ($p<0.01$). * $p<0.05$; ** $p<0.01$; n.s.: not statistically significant.

These results show that acute absence of all gangliosides by *in vitro* disruption of GM3, the only remaining type of ganglioside at dKO NMJs, at the one hand *increases* presynaptic neurotransmitter release by enhancing the amount of ACh released per nerve impulse but, on the other hand, *reduces* release by causing a more pronounced rundown during high-rate stimulation. It is yet unclear how gangliosides affect ACh release in this way. Possibly it involves a modulatory effect of the negative electrical

charges carried by sialic acid molecules of gangliosides on the presynaptic $\text{Ca}_v2.1$ channels that mediate neurotransmitter release at the NMJ (Kaja et al., 2007b). This type of channel has been shown to co-localize with gangliosides in lipid rafts (Taverna et al., 2004), which may facilitate interaction. Furthermore, experiments using anti-ganglioside antibodies suggest a relationship between gangliosides and $\text{Ca}_v2.1$ channels (Nakatani et al., 2009; Ortiz et al., 2001). $\text{Ca}_v2.1$ channel behaviour is one important determinant of EPP rundown level at high intensity use of NMJs (Kaja et al., 2005). Another indication for possible modulation of $\text{Ca}_v2.1$ channels by gangliosides is the reduction of 25 ms paired-pulse facilitation, because this phenomenon of short term synaptic plasticity is highly dependent on intraterminal accumulation of Ca^{2+} , mainly determined by influx levels and intracellular buffering capacity. Increased Ca^{2+} influx following neuraminidase treatment has also been shown in cardiac myocytes, possibly through opening of “leak” channels (Marengo et al., 1998). Alternatively, the sialic acids of gangliosides adjacent to presynaptic $\text{Ca}_v2.1$ channels at the motor nerve terminal might act to locally sequester extracellular Ca^{2+} , making it available upon high intensity use of the synapse in order to enable sustained transmitter release.

Although we are not aware of any sialylated proteins important for presynaptic transmitter release at mouse NMJs, we cannot completely exclude that (part) of the effects of neuraminidase treatment on synaptic transmission at the dKO NMJs was due to desialylation of such putative sialoproteins. Sialic acid removal from hippocampal voltage-gated Na^+ channels has been shown to increase action potential firing threshold (Isaev et al., 2007). However, such an effect occurring in phrenic nerves of our nerve-muscle preparations would be irrelevant because of the supramaximal stimulation we applied.

In view of the rather modest magnitude of the effects of total ganglioside ablation on ACh release, in combination with the increased initial quantal content, compensating for the reduced release at high rate stimulation, it is not to be expected that successful neuromuscular transmission becomes endangered. Synaptic transmission at the NMJ has a large safety factor (Wood and Slater, 2001), and in line with this we observed a sustained tetanic muscle contraction upon 40 Hz nerve stimulation when visually inspecting dKO hemi-diaphragms directly after the neuraminidase treatment.

In conclusion, we show that gangliosides play a role in transmitter release at the NMJ, albeit a rather modest one. Although an indispensable role for gangliosides in neurotransmission was hypothesized previously, they apparently are not absolutely required. The almost normal ACh release levels observed earlier at NMJs of GM2s-KO, GD3s-KO and dKO mice (Bullens et al., 2002; Zitman et al., 2008; Zitman et al., 2009) are likely due to compensatory effects of the remaining subsets of gangliosides, or GM3 ganglioside alone, respectively. It must be noted that we here studied synaptic effects of *acute* ablation of the last remaining ganglioside at dKO NMJs and that it cannot be excluded that *long-term* overall ganglioside deficiency has more severe synaptic effects. Such effects may underlie the severe neurodegenerative symptoms and premature death of transgenic mice lacking both GM2synthase and GM3synthase, which have permanently blocked *in vivo* synthesis of all gangliosides (Yamashita et al., 2005). In humans it was found that a lack of synthesis of the major gangliosides, due to an autosomal recessive truncation mutation in the *SIAT9* gene that encodes GM3-synthase, resulted in infantile-onset epilepsy associated with developmental stagnation and blindness (Simpson et al., 2004). The affected children show distorted reflexes and one case of startle myoclonus was observed. Neuromuscular transmission failure has not specifically been reported in these patients.

CHAPTER 3

**Pathophysiological roles of
anti-ganglioside antibodies at the
neuromuscular synapse**

CHAPTER 3.1

The role of complement and complement regulators in mediating motor nerve terminal injury in murine models of Guillain-Barré syndrome

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***Published in Journal of
Neuroimmunology 201 - 202 (2008) 172 - 182***

Abstract

Recent research into the Guillain-Barré syndromes (GBS) has focused on anti-ganglioside antibodies that correlate with specific clinical phenotypes. Our increasing understanding of the role of antibodies in mediating GBS has naturally focused our attention on complement involvement in the pathological procession. We have studied the axonal and glial components of the murine motor nerve terminal as a model site of antibody and complement mediated injury. Such studies are providing us with clear information on the molecular components underlying our clinicopathological model for GBS and have led us to the testing emerging complement therapeutics that are potentially suitable for human use.

Acknowledgements

Financial support was provided by the Wellcome Trust, Guillain-Barré Syndrome Support Group UK, Guillain-Barré Syndrome Foundation International and the Prinses Beatrix Fonds.

Introduction

Recent developments in our understanding of the Guillain-Barré syndromes (GBS) and variants including Miller Fisher syndrome (MFS) have divided them into distinct phenotypes with defining clinical, serological, electrophysiological and pathological features (Hughes and Cornblath, 2005). The major current research direction strongly suggests that antibody and complement mediated mechanisms are important in pathogenesis (Willison and Yuki, 2002; Yuki, 2001). It is clear that many different glycolipids act as autoantibody targets in peripheral nerve where their epitopes are spatially distributed in sites that reflect the clinical distribution of disease. Where antibody (of appropriate class and subclass) is found, complement follows, and there is also now extensive evidence that complement activation through the classical pathway is an important factor in driving pathology in animal models of GBS, supported by human pathological studies (Hafer-Macko et al., 1996a; Lu et al., 2000; Putzu et al., 2000; Wanschitz et al., 2003). This emphasis on humoral mechanisms sits in contrast to extensive past research based on predominantly T cell-mediated models of GBS, namely experimental allergic neuritis, in which clinical correlates have not been widely found to date, and in which complement would be expected to play a lesser role (Kieseier and Hartung, 2003). In this review and primary data report, we will focus on an area we have extensively researched in recent years, namely the emerging relationship between complement mediated distal motor nerve and motor nerve terminal injury, anti-GD1a, -GM1 and -GQ1b ganglioside antibodies, and motor axonal forms of GBS and MFS.

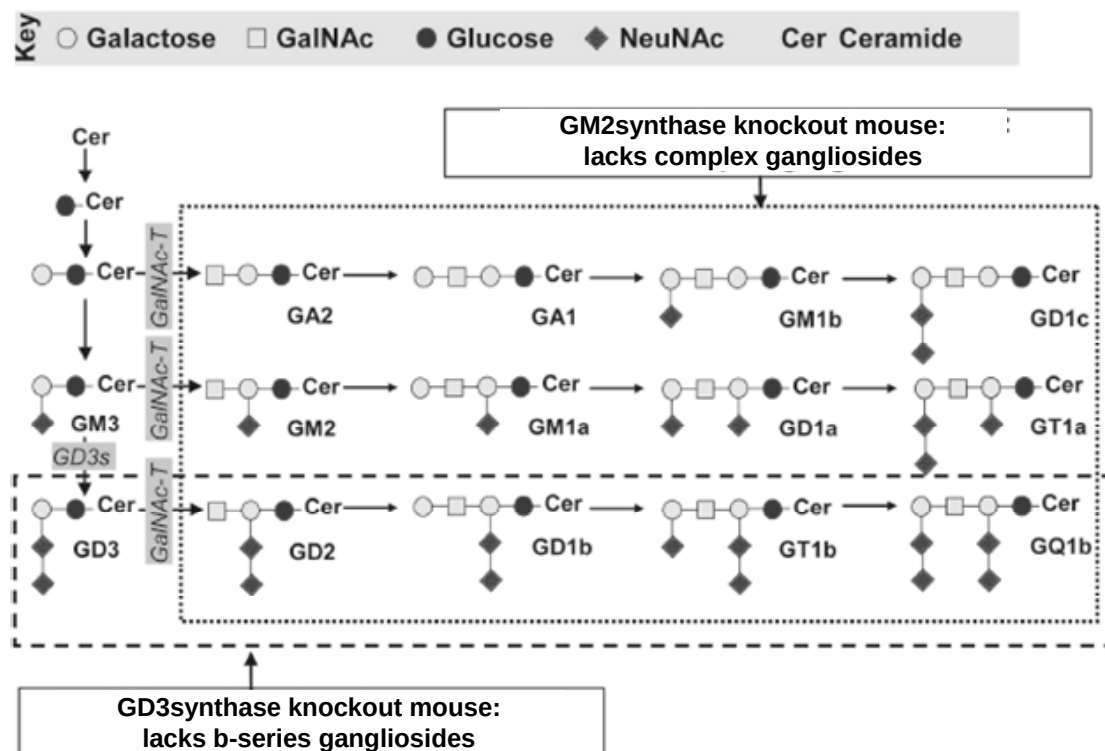


Figure 3.1. Schematic overview of the synthesis and composition of the ganglioside family

Indicated are the synthesis steps that are catalyzed by GM2synthase and GD3synthase and the ganglioside subgroups that are lacking in the respective knockout mice strains.

Our interest in the motor nerve terminal first arose through the knowledge that the pattern of weakness seen in MFS is similar to that seen in human botulism, a known disorder of presynaptic transmitter release at the neuromuscular junction (NMJ) (Lange et al., 2006). MFS comprises the clinical triad of ataxia, areflexia and ophthalmoplegia (Fisher, 1956). Interest in MFS was revived when Chiba and colleagues identified anti-GQ1b antibodies in the acute phase serum of MFS cases (Chiba et al., 1992). From this seminal observation, the measurement of anti-GQ1b antibodies in suspected cases of MFS is now widely used in clinical practice, with >90% sensitivity and specificity, akin to routine measurement of acetylcholine (ACh) receptor antibodies in myasthenia gravis.

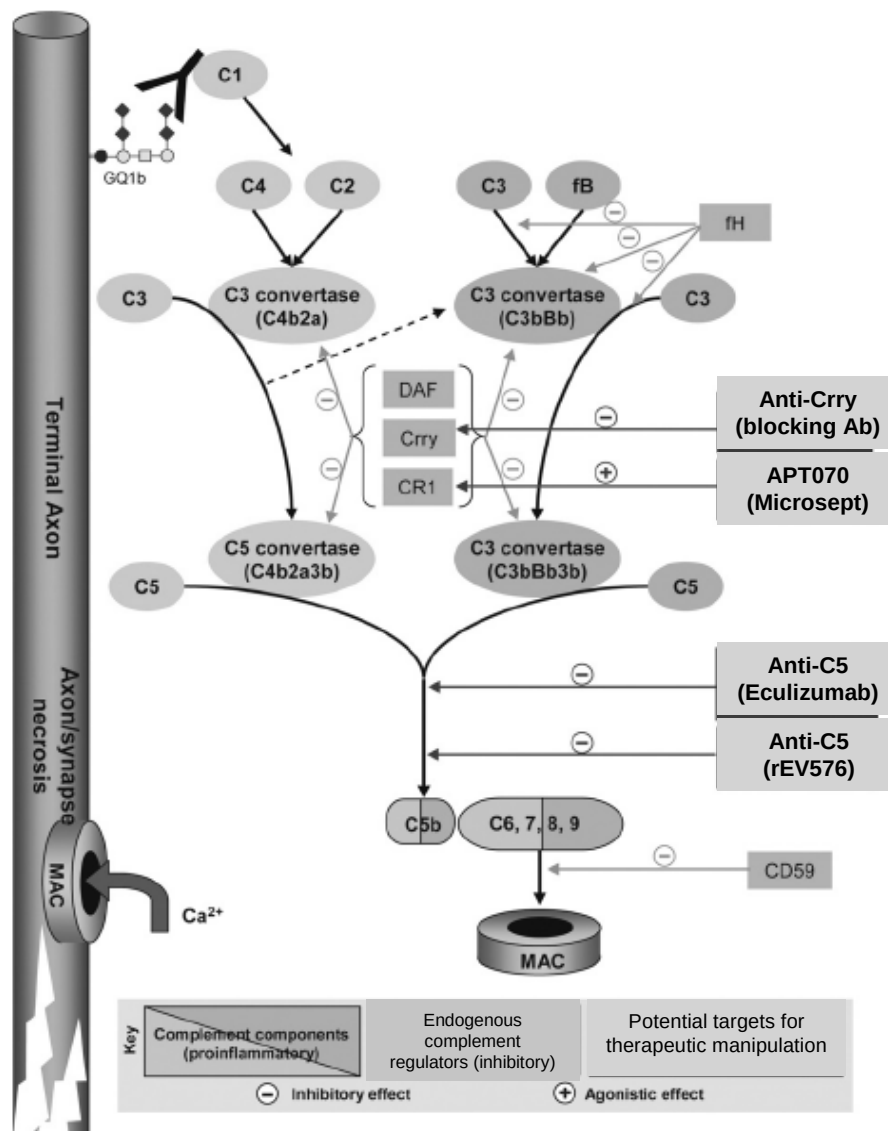


Figure 3.2.

A schematic diagram of potential therapeutic targets and strategies based on inhibition or removal of anti-ganglioside antibodies, inhibition of steps in complement activation through blocking antibodies or enhancement of complement regulation. Complement components in oval boxes: on the left, classical pathway; on the right, alternative pathway (C5b, C6, C7, C8, and C9 belong to both pathways).

Gangliosides are sialylated glycosphingolipids, concentrated in the outer leaflet of plasma membranes. They are highly enriched in the nervous system, including synaptic membranes and biosynthetically regulated by specific glycosyltransferases. A scheme is shown in Figure 3.1 (Sandhoff and Kolter, 2003). Since gangliosides are recognised as the presynaptic ectoacceptors for botulinum toxin binding, we anticipated that anti-ganglioside antibody-associated syndromes might also affect this site. Armed with this information, we established a collaboration with John Newsom-Davis and his colleagues in Oxford where we first demonstrated that the nerve terminal was indeed targeted by MFS-associated antibodies (Roberts et al., 1994). This landmark study inspired extensive subsequent work exploring the immunopathophysiology resulting from anti-ganglioside antibody and complement binding at the NMJ, as outlined in Figure 3.2. Many shared principles exist between anti-ganglioside antibody-associated syndromes and other antibody-mediated autoimmune diseases affecting the NMJ, including myasthenia gravis, Lambert-Eaton myasthenic syndrome and Isaac's syndrome (Vincent et al., 2006); in this commemorative paper we thus acknowledge the huge contribution of John Newsom-Davis and his group to our understanding of this field.

Anti-ganglioside antibody and complement effects on neuromuscular transmission

Following on from the early studies with the Newsom-Davis group (Benatar et al., 1997; Roberts et al., 1994; Roberts et al., 1995; Willison et al., 1996), we subsequently studied the effects of MFS sera and related anti-ganglioside human and murine monoclonal antibodies (mAbs) on NMJ electrophysiology in more detail in Leiden (Plomp et al., 1999). We first tested the effects of incubation of mouse hemidiaphragm preparations with anti-GQ1b-positive MFS sera. The sera induced a dramatic increase in the frequency (up to 300-fold at some NMJs) of spontaneously released ACh quanta, measured as miniature endplate potentials (MEPPs), without significantly altering their amplitudes. This was followed by block of evoked ACh release resulting in paralysis of the preparation, similar to the effects of the paralytic neurotoxin α -latrotoxin (α LTx) (Rohou et al., 2007). Incubation with total MFS IgG alone was without effect but subsequent treatment with freshly prepared normal human serum (NHS) readily induced high frequency MEPPs followed by transmission block, suggesting the involvement of complement in the phenomenon. Further subclass purification of IgG showed that α LTx-like activity co-eluted with anti-GQ1b activity in the same complement fixing IgG subclasses (IgG3 or IgG1), suggesting that these antibodies were the responsible factors. We demonstrated identical complement-dependent electrophysiological effects of anti-GQ1b-positive MFS/GBS sera in experiments using anti-GQ1b IgM mAbs that were derived from mice immunized with lipo-oligosaccharides which contained GD3/GT1a-like structures and originated from MFS/GBS-associated *C. jejuni* strains (Goodyear et al., 1999). In order to address whether the primary target of anti-GQ1b antibodies mediating the α LTx-like effect through complement activation were gangliosides or an unrelated (sialylated) antigen, for instance the α LTx receptor latrophilin, we exploited glycosyltransferase knockout mice that lack complex gangliosides (in collaboration with Furukawa). Through these studies we proved that the presynaptic complex ganglioside GQ1b must be the primary antigenic target of MFS-associated

antibodies and that the level of expression of gangliosides was a critical factor in influencing the extent of pathophysiology (Bullens et al., 2002). We have since demonstrated similar findings with anti-GD1a antibodies that occur in motor axonal forms of GBS (Goodfellow et al., 2005).

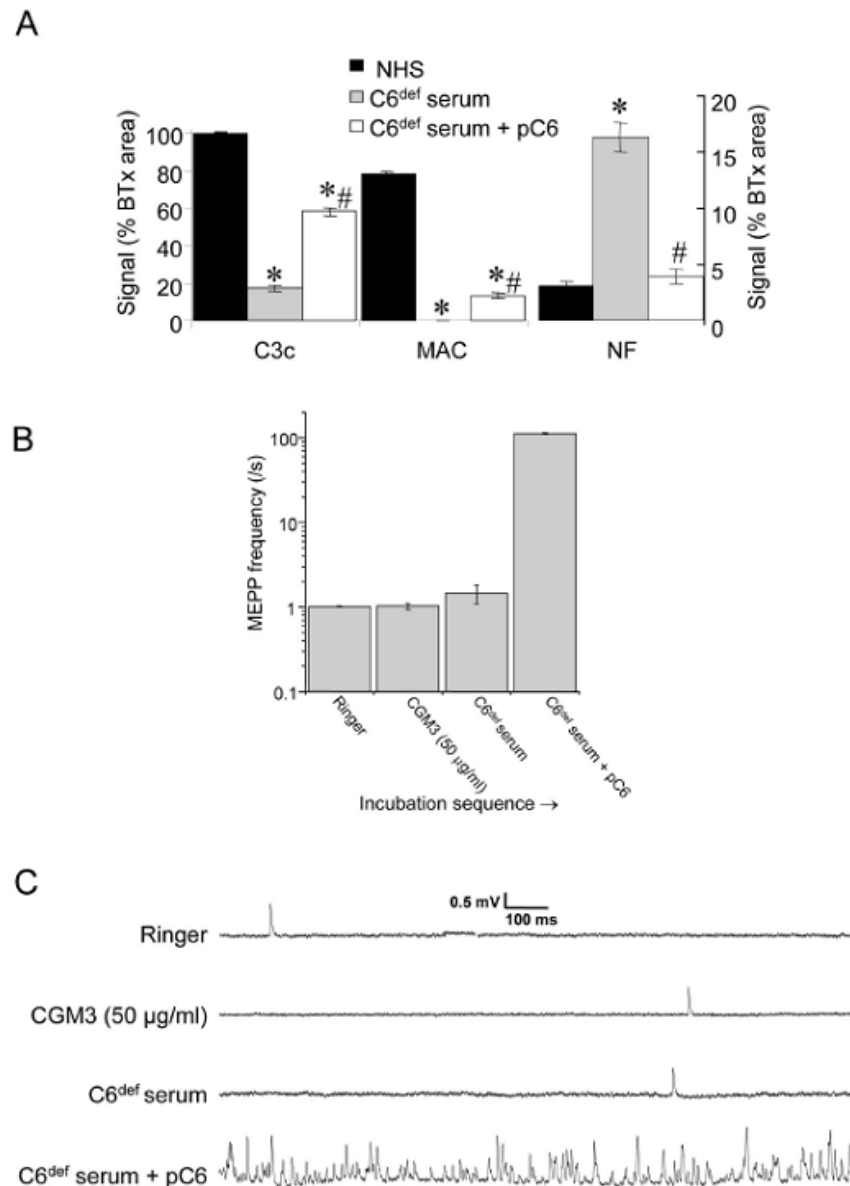


Figure 3.3.

A. Dependence of neurofilament (NF) loss on MAC deposition, assessed in total C6 deficiency (C6^{def} mouse with C6^{def} human serum). No MAC is formed (left axis) and there is no NF loss (right axis). Reconstitution of C6^{def} serum with purified C6 (pC6) leads to MAC deposits and NF loss. C3c is formed under both conditions (left axis). Student's two-tailed *t*-test: * = significantly different from NHS treated samples; # = significantly different from C6^{def} serum treated samples. (BTx = α -bungarotoxin). **B and C.** Electrophysiological analysis of NMJs under C6^{def} conditions: Hemidiaphragms of C6^{def} mice (*n*=2) were incubated serially in Ringer's medium, CGM3, C6^{def} serum and C6^{def} serum with added pC6. **B.** Spontaneous quantal ACh release at NMJs was measured as MEPPs with an intracellular microelectrode. MEPP frequency increased dramatically only when pC6 was added to the serum. Average MEPP frequency (*n*=2 muscles, 7-18 NMJs sampled per incubation). **C.** Typical examples of electrophysiological traces.

Immunopathological effects at the nerve terminal

In parallel with the electrophysiological studies described above, we assessed the immunohistological and morphological sequelae of anti-ganglioside antibody exposure to neuromuscular preparations *in vitro*, and to passively immunized mice. In topical immunostaining studies we had found that anti-GQ1b antibodies bound the NMJ (O'Hanlon et al., 2001; Willison and O'Hanlon, 1999) and thus also expected them to be deposited in passive immunization and exposure studies *in vivo* and *ex vivo* (Halstead et al., 2004). In contrast to the NMJ, we found little evidence of antibody penetration and binding to intramuscular nerve-bundles including nodes of Ranvier, despite reactive antigens being present in topical immunostaining studies. It is thus likely that antibody access to nerve fibres and nodes of Ranvier beyond the NMJ is limited by the blood nerve barrier, as we have previously observed for sheathed sciatic nerve preparations (Paparounas et al., 1999) and this finding reinforces our preference for understanding pathophysiological pathways using the NMJ as a model, rather than more proximal nerve sites.

Using confocal and immuno-electron microscopy (EM), we were able to distinguish the presynaptic neuronal elements, the perisynaptic Schwann cell (pSC) and the post-synaptic structures which comprise the NMJ, and the detailed localization of anti-ganglioside antibody and complement deposits at the NMJ using these methods has been examined. Depending upon the gangliosides examined and the strain of mouse used for the studies, extensive immunoglobulin deposits can be detected on presynaptic axonal membranes or pSC membranes, or both. At the fluorescence microscopy level, the former can be identified by proximity to α -bungarotoxin staining (marking ACh receptors and therewith the postsynaptic membrane), and the latter by observing that the labelling pattern extends around the nuclei of pSCs and co-locates with the Schwann cell specific marker, S100 in double staining studies. For anti-GQ1b antibodies, this has also been resolved by immuno-gold EM (Halstead et al., 2004).

At NMJs in mouse hemi-diaphragm preparation exposed to anti-GQ1b antibody in presence of NHS, complement products are deposited in a very similar distribution to anti-GQ1b immunoglobulins. Complement fragment C1q, C4, C3c, C5 and the pore-forming membrane attack complex (MAC, also termed C5b-9) can all be readily detected. In an extensive series of *ex vivo* and *in vivo* experiments using C6 complement component deficient sera and mice, we have demonstrated that the α LTx-like effects do not occur in the absence of MAC. Key features of these published data are shown in Figure 3.3. Whilst this does not preclude important pro-inflammatory roles for earlier complement components including C3a and C5a, it indicates that the major acute injury in this model is mediated by MAC. Extensive MAC-dependent cytoskeletal and mitochondrial changes occur concomitantly with the α LTx-like effect. The axonal cytoskeleton is severely affected, as assessed by loss of neurofilament (NF) and beta-tubulin immunoreactivity and abnormal EM appearance, when compared to controls. At the EM level, the intra-terminal mitochondria also show severe structural damage. Using this information, we have formulated a model of injury in which the dominant event is uncontrolled Ca^{2+} influx through MAC pores into the nerve terminal, resulting in uncontrolled ACh exocytosis, calpain activation with NF degradation and mitochondrial injury (O'Hanlon et al., 2003). This model is summarized in Figure 3.4.

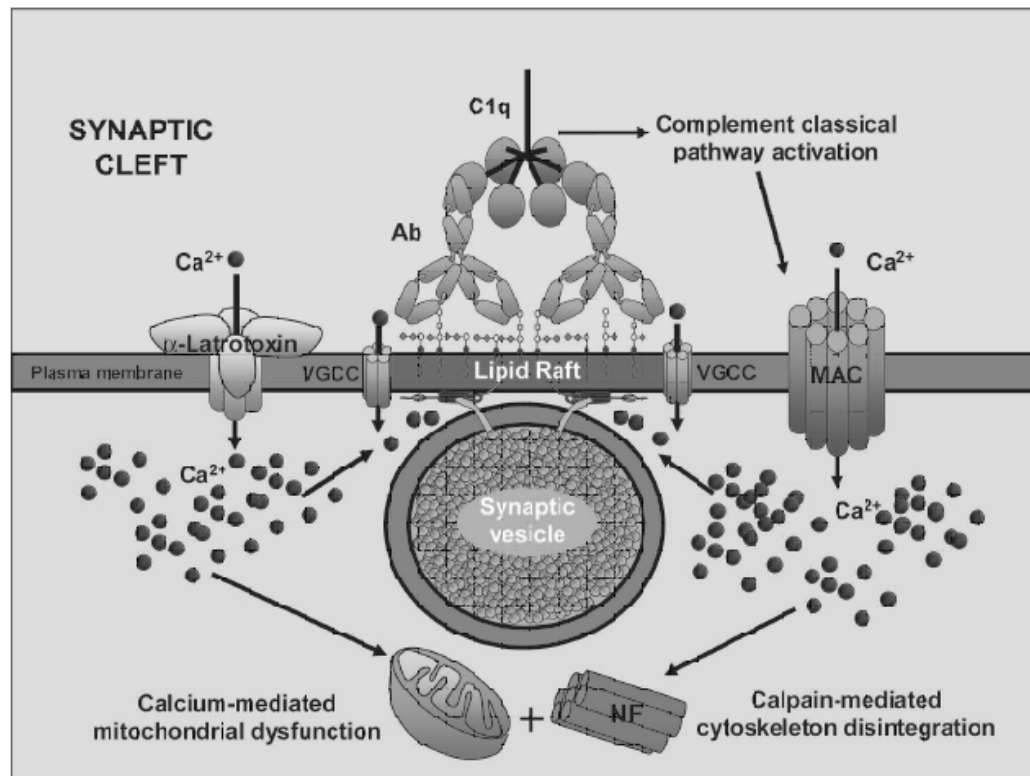


Figure 3.4. A schematic diagram of the events occurring at nerve terminals in experimental nerve-muscle preparations exposed to anti-ganglioside antibodies in the presence of complement. Exocytosis is normally initiated by highly regulated Ca^{2+} entry through voltage-gated Ca^{2+} channels (VGCC) that activates the adjacent exocytotic protein complex and initiates synaptic vesicle fusion with the pre-synaptic membrane. When MAC pores (on the right) are deposited in the pre-synaptic membrane, unregulated Ca^{2+} entry triggers massive, uncontrolled exocytosis and Ca^{2+} /calpain mediated intra-terminal injury, including cytoskeletal degradation, assessed by measuring neurofilament (NF) levels, and mitochondrial injury. The pore forming toxin, α -latrotoxin (on the left), is believed to act in part through a similar mechanism, allowing unregulated Ca^{2+} influx with similar electrophysiological and morphological sequelae to those resulting from MAC pores.

The experimental data described above is derived from mouse modelling and what remains unclear is the extent to which the distal motor axon is a site of injury relevant to human disease. The availability of (motor point) muscle biopsy tissue from human cases at the appropriate time point in the clinical course is practically very limited, either in MFS or in GBS, and even if it were available the interpretation is likely to be confounded by the co-occurrence of Wallerian degeneration arising from more proximal axonal injury. Direct electrophysiological assessment of human distal motor axonal function is relatively crude and imaging this site is not feasible. These factors make it difficult to assess the validity of these mouse data as a direct model of the human disease. However, indirect evidence indicates that the distal motor axon is an affected site in axonal forms of GBS (Ho et al., 1997; Kuwabara et al., 2003), along with other sites including nerve roots and trunks. Furthermore, several case studies have supplied clinical electrophysiological indications for NMJ defects in MFS (Lange et al., 2006; Lo et al., 2006; Sartucci et al., 2005; Uncini and Lugaresi, 1999; Wirguin et al., 2002). Irrespective of the human clinical relevance of the distal motor axon, the principles governing the effects of antibody binding with complement fixation at ganglioside-rich membrane sites are likely to be applicable to axonal and glial membranes in general.

The influence of endogenous complement regulators on motor nerve terminal injury

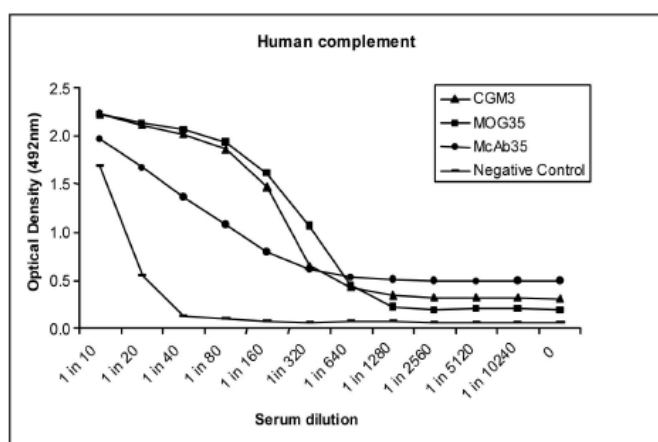
One perplexing issues surrounding our model of anti-GQ1b and MAC mediated injury is its dependence upon an exogenous, heterologous source of complement to induce the neuropathic lesions in mouse tissues; in our case we achieve this with NHS. Previously, we have attempted to generate a model by intraperitoneal injection of anti-ganglioside mAb, relying on mouse serum as a source of complement; whilst we could demonstrate clear antibody deposits overlying presynaptic nerve terminals accompanied by subtle electrophysiological abnormalities, no behavioural defects were observed (Willison et al., 1996). In subsequent studies we have sought mouse complement deposits and found them to be very sparse. We considered factors that might influence this failure of anti-ganglioside antibodies to activate mouse complement on the presynaptic membrane. To this end, we have conducted a range of studies in parallel with a positive control study in the form of a complement fixing anti-acetylcholine receptor (AChR) rat mAb (McAb3, kindly provided by Vanda Lennon, Mayo Clinic), that is known to induce complement-dependent experimental myasthenia gravis on passive immunization in mice in the absence of a heterologous complement source. As this antibody binds on the postsynaptic membrane, only ~50 nM distant from the anti-ganglioside mAb binding region on the presynaptic membrane, it would appear to be a good control in terms of the diffusion dynamics of antibody and complement components from the circulation.

In this comparative study we first confirmed that all the mouse mAbs we were using in passive immunization studies were able to fix mouse complement in an equivalent manner to the rat anti-AChR mAb, McAb3 (primary data, Figure 3.5). There are variations both the complement fixing abilities of different antibodies and in complement activity in laboratory mice strains that confound these issues (Rice, 1950). For example, the low haemolytic capacity of mouse serum may be in part due to the inability of the C4b subunit of the classical pathway C5 convertase to bind C5 (Ebanks and Isenman, 1996). Nevertheless, these data show that the mouse anti-glycolipid mAbs used in our studies are as able to effectively fix mouse complement as a known myasthenia gravis-inducing pathogenic antibody reactive to AChR, using C57bl/6 serum in solid-phase ELISA studies, conducted according to Zwirner with minor modifications (Zwirner et al., 1998). From this starting point it seems very likely that unknown inhibitory factors must present in the presynaptic membrane that differ from those in the postsynaptic membrane. Candidate molecules include the murine complement regulators complement receptor-1 related gene/protein Y (Crry), CD59a, decay accelerating factor 1 (DAF1, CD55) and Factor H that are widely distributed and limit the formation of activated complement products (Figure 3.2). We can clearly observe CD59a by immunofluorescence microscopy in the presynaptic axonal and pSC membranes using mouse anti-CD59 mAb 7A6 (Donev et al., 2008). This is illustrated in Figure 3.6 (primary data), using transgenic mice provided in collaboration with the Thompson laboratory that also express the endogenously fluorescent proteins cyan fluorescent protein (CFP) and green fluorescent protein (GFP) respectively in the cytoplasm of these NMJ components (Feng et al., 2000; Zuo et al., 2004). We have also previously shown that CD59a deficiency plays an aggravating role in our model of injury, although in these studies the complement source was provided by NHS, a heterologous source of complement whose degree of regulation by murine CD59 is not clearly delineated (Halstead et al., 2004). The

expression of membrane-associated DAF1 and Cr3 has been more difficult to detect at this site by immunohistology.

Using CD59a and DAF1 deficient mice in the McAb3 passive immunization model of myasthenia gravis, exacerbation of pathology has clearly been shown in comparison to wildtype, the presumption therefore being that both CD59a and DAF1 are present in the postsynaptic membrane (Lin et al., 2002; Morgan et al., 2006). In collaboration with the Medof laboratory, our passive immunization experiments conducted to date in CD59a/DAF1 double knockout mice with anti-ganglioside antibody (in the *absence* of NHS as a source of complement) have not clearly demonstrated any major clinical or morphological differences when compared with wildtype controls. This is in clear contrast to the situation with the myasthenia gravis model, both from the published data and from experiments we have also conducted in Glasgow in parallel by way of control. We are currently assessing these double knockout mice for subtle electrophysiological abnormalities (Zitman and Halstead, unpublished observations). Although we do observe an increase in C3c deposits in these double knockout mice, one of the limitations in detailing subtle quantitative changes in MAC levels in these studies has been the absence of suitable mouse MAC probes producing the very low backgrounds needed for our quantitative analytical methods, in comparison with the widely available reagents for human MAC. We therefore cannot conclude that no MAC is formed, but nevertheless cannot observe it at significant levels compared with control tissue.

A



B

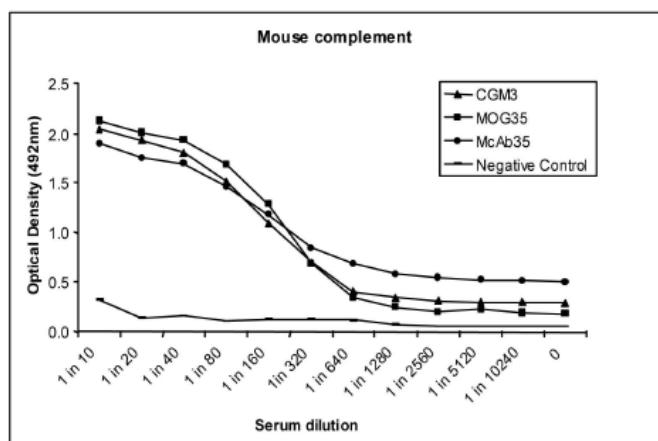


Figure 3.5. Complement activation ELISA

Activation of complement in human and mouse serum was compared between 3 antibodies of different specificities, species and subclasses. MAbs CGM3 (mouse IgM), MOG35 (mouse IgG2b) and McAb3 (rat IgG) were captured on microtitre plates and serial dilutions of human (A) or mouse serum (B) was applied. The complement activation capacity of the antibodies was assessed by detecting the presence of activated C3 with an anti-C3c antibody (Dako). Mouse mAbs CGM3 and MOG35 had comparable levels of human C3, while rat mAb McAb3 fixed human C3 to a slighter lesser degree. All 3 mAbs activated mouse C3 to similar levels. Method based on Zwirner (Zwirner et al., 1998).

Many other complement regulators exist, and cross-regulation across species is complex and controversial (Morgan et al., 2005). Our previous studies using NHS in passively immunized mice have demonstrated activation of the complement cascade via the classical pathway (Halstead et al., 2004); however classical pathway deposited C3b can also serve as a nidus for the alternative pathway positive feedback loop, thereby resulting in the further amplification of the cascade (Fearon and Austen, 1980). A key regulator of the alternative pathway is the soluble glycoprotein Factor H (fH), which has both decay accelerating activity against the alternative pathway C3 convertase (Pangburn and Muller-Eberhard, 1978; Weiler et al., 1976) and cofactor activity for Factor I mediated C3b cleavage (Pangburn et al., 1977). Sialic acid enriched membrane (in our case the presynaptic membrane) has also been shown to prevent alternative pathway activation, by increasing the affinity of fH for C3b (Fearon, 1978; Kazatchkine et al., 1979). Indeed, we see heavy deposits of fH at NMJs as detected with polyclonal sheep anti-fH antibody (Abcam; primary data, Figure 3.7), but only in the presence of activated complement in our anti-ganglioside antibody and NHS passive immunization model. The amount of fH deposited in other models has not been reported in detail.

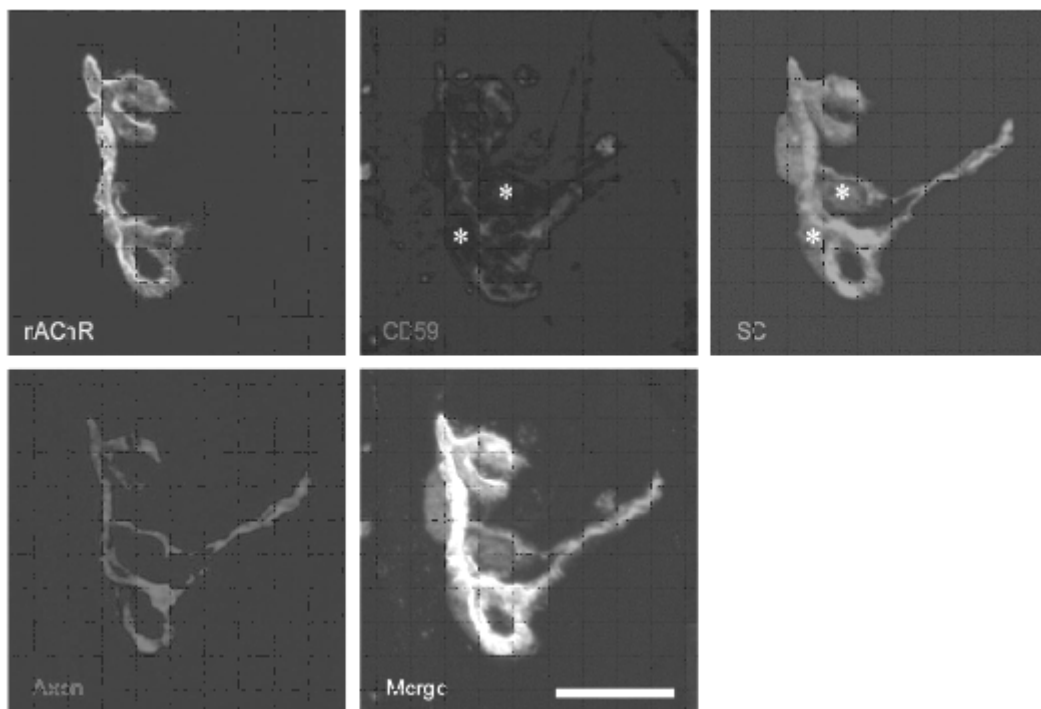


Figure 3.6. Illustrative immunofluorescence image of whole-mount triangularis sternae nerve-muscle preparation demonstrating the presence of CD59a at the NMJ

Transgenic mice which express cytoplasmic green fluorescent protein in their Schwann cells (SC; upper right panel) and cyan fluorescent protein in their axons (Axon; lower left panel) were stained with α -bungarotoxin to delineate their postsynaptic nicotinic acetylcholine receptors (nAChR; upper left panel) and anti-mouse CD59 (upper middle panel). CD59 is present on both the motor axon and perisynaptic Schwann cells (asterisk).

Scale bar: 20 μ m.

We therefore hypothesize that the failure of endogenous mouse complement to generate a pathological lesion in our anti-ganglioside antibody passive immunization model results from the relative inability of the classical pathway C5 convertase to cleave C5 and progress to MAC formation in the presynaptic membrane. In other sites, for example the postsynaptic membrane that is targeted in myasthenia gravis, this might be overcome by amplification of the alternative pathway loop which is activated through the deposition of classical pathway C3b. On the presynaptic membrane, where anti-ganglioside antibodies bind their sialic-acid containing ganglioside targets in glycolipid-enriched microdomains (functional rafts), it is possible that the alternative pathway loop might be especially highly regulated by fH because of the rich sialic acid environment at this site. fH could thereby more tightly regulate the progression of the cascade to MAC in this site compared with other sites where autoantibodies bind protein targets such as AChR, i.e. the postsynaptic membrane. This hypothetical model remains consistent with our unpublished observation of a rise in C3c levels in the DAF1/CD59a double knockout as the relative deficiency in the classical pathway C5 convertase (component C4b), along with the fH regulation discussed above, follows after the step at which DAF1 exerts its C3 convertase regulation. The implication of this is that the deficiency of CD59a, which regulates at the C5b-8 level, does not overcome the inhibitory events earlier in the pathway as there are no or very low levels of activation products for it to regulate. Further studies are needed to address these complex issues surrounding the activation and regulation of complement in different sites and species in models of antibody mediated autoimmune disease.

Therapeutic strategies for the attenuation of complement activation

As a result of the apparent inability of anti-glycolipid antibodies to fix mouse complement in the presynaptic membrane, our studies have been pursued with NHS as a source of heterologous complement. The mechanism(s) by which activation of human complement is able to proceed, where mouse complement fails is unknown, but presumably relates to a relative inefficiency of heterologous regulation. In our situation this species-specific effect has the fortuitous benefit of allowing us to test any complement therapeutics in a model system in which the complement component proceeding to MAC has essentially been “humanized”, and thus has added relevance to preclinical testing of agents that might be suitable for human clinical trials. Although anti-ganglioside antibodies may exert some of their paralytic effects directly (independent of complement) as shown in an extensive series of studies from the Toyka group (Buchwald et al., 2001), tissue bound antibody of the appropriate class as found in GBS (human IgG1 and IgG3) inevitably fixes complement to exacerbate injury unless complement regulation is highly active. Thus, one key approach that might be expected to suppress disease activity in anti-ganglioside antibody-mediated neuropathy is the suppression of complement activation. Since it is very clear that complement activation with MAC formation drives neural membrane injury in anti-ganglioside antibody treated mouse tissue and in rabbit models of acute motor axonal neuropathy (Susuki et al., 2007a), it would appear likely that blocking MAC formation locally should prevent MAC-dependent tissue injury, even if anti-ganglioside antibodies are deposited in neuronal or myelin membranes. We recently

investigated this using the complement inhibitor APT070 that contains the C3/C5 convertase inhibiting region of complement receptor 1 and a membrane-localizing peptide that allows it to inhibit complement accumulation. APT070 pretreatment completely abrogated MAC formation and acute tissue injury of anti-ganglioside antibody immunized mice both *ex vivo* and *in vivo* (Halstead et al., 2005a).

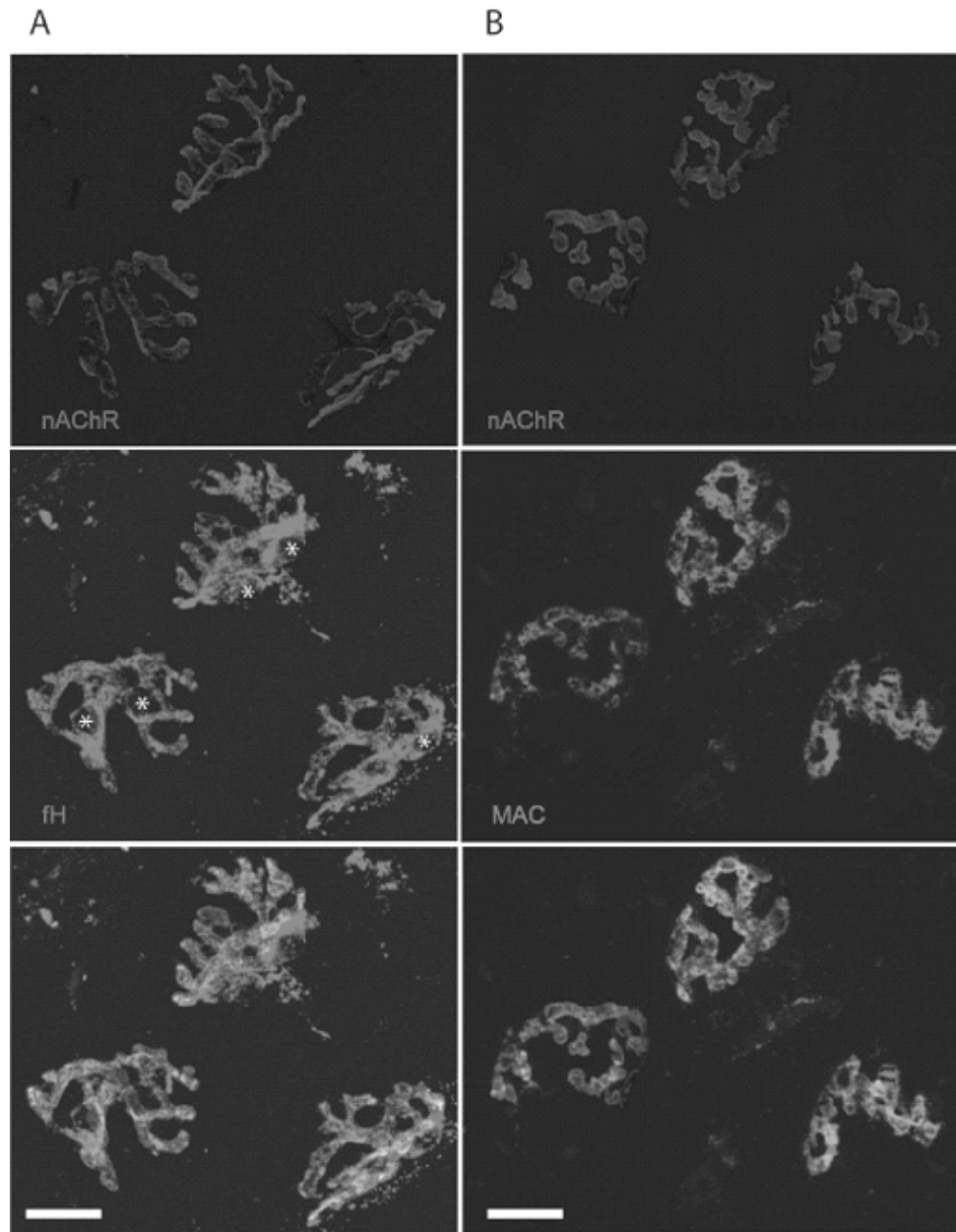


Figure 3.7. Illustrative immunofluorescence images of mouse triangularis sternae nerve-muscle organ bath preparations *in vitro*, demonstrating complement deposition at the NMJ

Live mouse tissue was incubated with the anti-ganglioside IgM mAb, CGM3, followed by 40% normal human serum (NHS) as a source of complement, according to Halstead (Halstead et al., 2005b). **A.** Postsynaptic nicotinic acetylcholine receptors (nAChR) delineate the NMJ (top). Factor H (fH, middle) is extensively deposited present on the terminal motor axon and perisynaptic Schwann cells (asterisk). **B.** In this model, the complement cascade in human serum is activated, culminating in human membrane attack complex (MAC; middle) deposits over presynaptic neuronal and Schwann cell membranes.

Lower panels show merged views. Scale bar: 20µm

We have also assessed the efficacy of the humanized anti-C5 mAb eculizumab, which neutralizes C5 and thereby blocks the formation of human C5a and C5b-9, in preventing the anti-ganglioside antibody and complement-mediated motor nerve terminal injury (Halstead et al., 2008a). In these studies, eculizumab completely prevented electrophysiological and structural lesions at anti-GQ1b antibody pre-incubated NMJs *in vitro* when using NHS as a complement source. In our *in vivo* mouse model of MFS generated through intraperitoneal injection of anti-GQ1b antibody and NHS, mice developed respiratory paralysis due to transmission block at diaphragm NMJs, resulting from anti-GQ1b antibody binding and complement activation. Intravenous injection of eculizumab effectively prevented respiratory paralysis and associated functional and morphological hallmarks of terminal motor neuropathy. Very recently we have also assessed whether the complement C5 inhibiting recombinant protein, rEV576, derived from tick saliva (Hepburn et al., 2007), is able to prevent neural injury in the mouse model for MFS (Halstead et al., 2008b). Again, mouse hemi-diaphragm preparations were treated with anti-GQ1b antibody and NHS as a source of complement with added rEV576 or control protein. In control tissue C3c and MAC were deposited at NMJs, along with terminal motor axonal NF degradation, pSC injury and electrophysiological and functional impairment. In tissue treated with rEV576, all these indicators of motor neuronal damage were absent, except for the presence of C3c, indicating effective inhibition of C5. These results demonstrate that rEV576 is a further agent that effectively prevents development of neuronal and pSC damage in experimental murine neuropathy.

Conclusions

It is well established that MAC is present in human GBS nerve biopsy and autopsy material and that this is likely to be an important step in generating pathology and functional loss. It is also very clear that complement activation with MAC formation drives neural membrane injury in our models of anti-ganglioside antibody treated mouse tissue. Since MAC is a convergence pathway in many antibody and complement-mediated diseases it would seem an excellent target for human therapeutic trials. Whilst our studies focus on ganglioside targets in the presynaptic membrane, other presynaptic targets and the postsynaptic membrane that is targeted by antibody in myasthenia gravis are also important candidates for therapeutic complement inhibition. The use of complement inhibition trials in clinical practice is thus eagerly awaited across several disease areas of antibody-mediated clinical neuroimmunology at the NMJ, to which the pioneering contribution of John Newsom-Davis was substantial.

CHAPTER 3.2

Eculizumab prevents anti-ganglioside antibody-mediated neuropathy in a murine model

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Published in Brain 131 (2008), 1197 - 1208

Abstract

Anti-GQ1b ganglioside antibodies are the serological hallmark of the Miller Fisher syndrome (MFS) variant of the paralytic neuropathy, Guillain-Barré syndrome, and believed to be the principal pathogenic mediators of the disease. In support of this, we previously showed in an *in vitro* mouse model of MFS that anti-GQ1b antibodies were able to bind and disrupt presynaptic motor nerve terminals at the neuromuscular junction (NMJ) as one of their target sites, thereby causing muscle paralysis. This injury only occurred through activation of complement, culminating in the formation and deposition of membrane attack complex (MAC, C5b-9) in nerve membranes. Since this step is crucial to the neuropathic process and an important convergence point for antibody and complement mediated membrane injury in general, it forms an attractive pharmacotherapeutic target. Here, we assessed the efficacy of the humanized monoclonal antibody eculizumab, which blocks the formation of human C5a and C5b-9, in preventing the immune-mediated motor neuropathy exemplified in this model. Eculizumab completely prevented electrophysiological and structural lesions at anti-GQ1b antibody pre-incubated NMJs *in vitro* when using normal human serum as a complement source. In a novel *in vivo* mouse model of MFS generated through intraperitoneal injection of anti-GQ1b antibody and normal human serum, mice developed respiratory paralysis due to transmission block at diaphragm NMJs, resulting from anti-GQ1b antibody binding and complement activation. Intravenous injection of eculizumab effectively prevented respiratory paralysis and associated functional and morphological hallmarks of terminal motor neuropathy. We show that eculizumab protects against complement-mediated damage in murine MFS, providing the rationale for undertaking clinical trials in MFS and other antibody-mediated neuropathies in which complement activation is believed to be involved.

Acknowledgements

This work was supported by grants from the Wellcome Trust (#077041/Z/05/Z, to HJW) and the Prinses Beatrix Fonds (#MAR04-0213, to JJP).

Introduction

Miller Fisher syndrome (MFS) is characterized by the triad of ataxia, areflexia and ophthalmoplegia (Fisher, 1956) and is a clinical variant of the post-infectious paralytic neuropathy, Guillain-Barré syndrome (GBS) (Hughes and Cornblath, 2005). MFS is associated with anti-GQ1b ganglioside antibodies that are considered to be the predominant pathogenic factor and may arise through molecular mimicry during a preceding infection with *Campylobacter jejuni* or other microbes bearing GQ1b mimics in their lipooligosaccharide (Goodyear et al., 1999; Willison and O'Hanlon, 1999; Yuki, 2001; Bowes et al., 2002). Gangliosides are a family of sialylated glycosphingolipids that are enriched in neuronal cell membranes (Ledeen, 1985). Ophthalmoplegia in MFS is thought to be related to the relatively high GQ1b ganglioside level in oculomotor nerves, forming a preferential antigenic target for anti-GQ1b antibodies (Chiba et al., 1993). Furthermore, anti-GQ1b antibody binding to the presynaptic motor nerve terminal compartment of the neuromuscular junction (NMJ), causing synaptic transmission block, has been proposed by us and others from animal studies (Roberts et al., 1994; Buchwald et al., 1995; Plomp et al., 1999). This idea was based on data that 1) botulism, an established NMJ disorder, is an important differential diagnosis for MFS, 2) cholinergic synapses are highly enriched in gangliosides (Schwarz and Futerman, 1996) and 3) NMJs are easily accessible by antibodies because there is no blood-nerve barrier. A number of case studies have since supplied clinical electrophysiological indications for NMJ dysfunction in MFS (Uncini and Lugaesi, 1999; Wirguin et al., 2002; Sartucci et al., 2005; Lange et al., 2006; Lo et al., 2006).

We have developed an *in vitro* experimental mouse model for MFS in which NMJs of the diaphragm-phrenic nerve preparation are exposed to mouse or human (monoclonal) anti-GQ1b antibodies or anti-GQ1b positive patient sera. The model has been extensively characterized in electrophysiological, morphological and functional studies (Goodyear et al., 1999; Plomp et al., 1999; Bullens et al., 2000; O'Hanlon et al., 2001; Jacobs et al., 2002; Halstead et al., 2004). These studies demonstrate that both human and mouse anti-GQ1b polyclonal antisera and monoclonal antibodies bind to the presynaptic motor nerve ending and activate the complement cascade, culminating in the formation of membrane attack complex (MAC, C5b-9). This pore-forming complex damages the presynaptic membrane, causing uncontrolled neurotransmitter release, ultrastructural destruction of the nerve terminal, and finally, blockade of neuromuscular transmission with resultant paralysis.

Tissue-bound antibody of the appropriate class fixes complement and experimental neuropathological effects in our MFS model are strictly dependent on complement activation (Plomp et al., 1999; Bullens et al., 2000; Halstead et al., 2004). Furthermore, there is evidence of complement involvement in variants of GBS (Sanders et al., 1986; Koski et al., 1987; Hartung et al., 1987; Hafer-Macko et al., 1996a). In view of the above, the complement system is an attractive target for pharmacotherapeutic intervention in MFS, and has led us to examine inhibitors of complement activation as potential therapies for the disease in humans, and by inference, also for pathophysiologically similar forms of GBS and other autoimmune neuropathies, including chronic inflammatory demyelinating polyneuropathy and multifocal motor neuropathy (Halstead et al., 2005a). Eculizumab (Soliris, Alexion Pharmaceuticals) is a humanized monoclonal antibody (mAb) that specifically binds to and blocks the cleavage of human complement component C5, thereby preventing

the formation of the proinflammatory and cell lytic molecules C5a and C5b (Thomas et al., 1996). Targeted blockade of complement at C5 preserves the earlier immunoprotective effects of complement activation (e.g. C3b opsonisation and phagocytosis). In addition to protecting against antibody mediated effects, complement inhibitors also have the potential to protect against secondary events such as those occurring in Wallerian degeneration (Ramaglia et al., 2007). Eculizumab has recently been approved to treat paroxysmal nocturnal haemoglobinuria, a genetic disorder with complement-mediated haemolysis as the primary clinical manifestation (Hillmen et al., 2006).

Here, we investigated the possible benefit of eculizumab in immune-mediated neuropathy by assessing its effects in the existing *in vitro* model for MFS and in a newly generated *in vivo* mouse model with paralytic symptoms. Despite extensive *in vitro* data, characterization of an anti-GQ1b antibody mediated clinical phenotype in active or passive immunisation models in the living mouse has not been achieved to date. Here we have used plethysmography to quantify respiratory function in living mice passively immunized with anti-GQ1b antibody and human complement through intraperitoneal injection and demonstrate the rapid development of a severe respiratory phenotype. Eculizumab completely prevented the presynaptic electrophysiological dysfunction and structural damage at *in vitro* NMJs that had been pre-incubated with anti-GQ1b mAb and efficiently prevented *in vivo* respiratory paralysis in the newly developed *in vivo* MFS mouse model.

Methods

Mice

Male Balb/c mice (3-6 weeks old, 10-25 g) were obtained from Harlan (UK or NL). In some muscle contraction experiments (see below) male and female GM2synthase *null*-mutant mice (Bullens et al., 2002) were used at 12-22 weeks of age (19-37 g). All animal experiments were carried out in accordance to UK Home Office guidelines (UK PPL60/3096), Dutch law and Glasgow and Leiden University guidelines.

Monoclonal antibodies and normal human serum

The IgM anti-GQ1b ganglioside mAb, CGM3 was derived from mice inoculated with a GT1a-bearing *C. jejuni* lipooligosaccharide (Goodyear et al., 1999). CGM3 reacts with gangliosides GQ1b, GD3 and GT1a that all share the terminal disialylgalactose structure. Previous *in vitro* studies have shown that CGM3 has similar ganglioside specificity and induces identical complement-dependent pathogenic effects as human MFS sera (Goodyear et al., 1999; Plomp et al., 1999; Bullens et al., 2000; Jacobs et al., 2002; Jacobs et al., 2003). The advantage of using a mAb for these studies is that, unlike human sera, it is a quantitatively unlimited and homogeneous resource that can be fully quantified and characterized and prepared as a stock solution for long-term use during the course of the experiments. CGM3 concentration was measured using quantitative ELISA (Bethyl Laboratories). Normal human serum (NHS) was taken from a single donor stock that had been freshly frozen and stored in multiple aliquots at -70 °C to preserve complement activity. Prior to experimental use, CGM3 and NHS were dialyzed for 24 h at 4 °C against Ringer's solution (116mM NaCl, 4.5mM KCl, 1mM MgCl₂, 2mM CaCl₂, 1mM NaH₂PO₄, 23mM NaHCO₃, 11mM glucose, pH 7.4), pre-gassed with 95% O₂ /5% CO₂. The humanized anti-human C5 mAb eculizumab

and a non-specific isotype-matched control mAb ALXN3300 were obtained from Alexion Pharmaceuticals and stored at 4 °C. Eculizumab binds plasma C5 only and thereby prevents its cleavage into C5a and C5b in the liquid-phase, but does not bind to membrane bound C5b as part of the MAC complex. The anti-C5 antibody is therefore not deposited in the tissue. Eculizumab is also monospecific for human C5, and does not bind to C5 from other species, including the mouse. Thus in this model, eculizumab is only capable of neutralizing the heterologous C5 in the human serum added as a final step in the *in vitro* exposure and passive immunization paradigms.

***In vitro* MFS model**

The *in vitro* model for MFS using CGM3 and NHS has been described before (Goodyear et al., 1999; O'Hanlon et al., 2001). In short, mouse hemi-diaphragms with phrenic nerve attached (or triangularis sterni muscle in some cases for illustrative NMJ immunohistology) were dissected and mounted in Ringer's medium at room temperature (20-22 °C). Untreated small control sections were removed from each muscle preparation prior to any incubation and snap frozen on dry ice for subsequent baseline immunohistological analysis. Muscles were incubated with CGM3 (50 µg/ml) for 2-2.5 h at 32 °C, then for 30 min at 4 °C and then equilibrated for 10 min at room temperature, rinsed in Ringer's medium and subsequently exposed to 33 or 40% NHS in Ringer's medium for 1 h at room temperature. Eculizumab (100 µg/ml) or the control mAb (100 µg/ml) was mixed with NHS 10 min prior to the incubation of the muscle preparation. *In vitro* electrophysiological measurements at the NMJ (see below) and scoring of muscle fibre twitching, a hallmark of uncontrolled transmitter release at NMJs in this model (O'Hanlon et al., 2001; Jacobs et al., 2002), were performed before and after CGM3 incubation, and during the 1 h NHS incubation. Muscle strips were frozen on dry ice and stored at -20 °C, both before and after incubation with NHS. NMJs and terminal motor axons were assessed for levels of IgM, C3c, MAC, neurofilament (NF) and for perisynaptic Schwann cell (pSC) viability (see below).

***In vivo* MFS model**

Balb/c mice (3-4 weeks old, 10-15 g) were injected intraperitoneally with 1.5 mg CGM3, followed 16 h later by an intraperitoneal injection of 0.5 ml 100% NHS. Mice were observed for a further 4-6 h and analyzed with whole-body plethysmography (see below) and grip strength testing as described before (Kaja et al., 2007a). The mice were then killed by CO₂ asphyxiation and hemi-diaphragm muscle tissue was dissected and analyzed in electrophysiological and muscle contraction experiments (see below) or further processed for immunohistological analyses (see below). The effect of eculizumab on the *in vivo* symptoms and the subsequently analyzed electrophysiological, functional and histological lesions was tested in this model by injecting 200 µg eculizumab or control mAb in the tail vein, shortly before intraperitoneal NHS injection. Initial dose-range finding experiments (data not shown) indicated that eculizumab at doses of >50 µg/mouse protected against neuropathy.

Plethysmography

Non-invasive whole-body plethysmography (EMMS) was employed to demonstrate changes in breathing parameters in *in vivo* experiments. Baseline data was collected prior to onset of experiment. Mice were injected with CGM3 followed by NHS with eculizumab or control mAb 16 h later (protocol as above) and monitored continuously

for 6 h. Flow derived parameters of breath frequency and tidal volume were collected from 25 accepted breaths and averaged over 1 h periods.

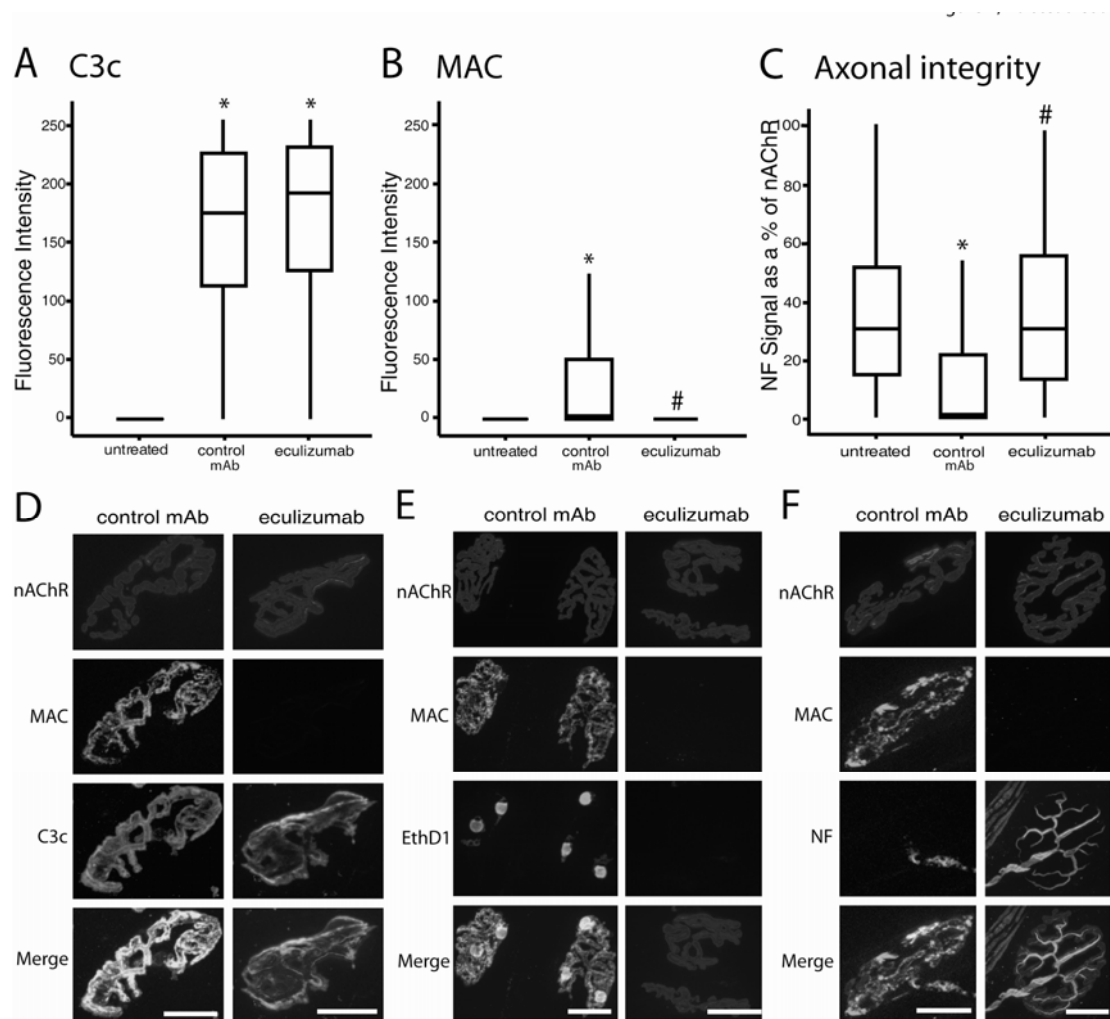


Figure 3.8. Immunohistological demonstration of the protective effect of eculizumab against MAC deposition and terminal motor axonopathy at NMJs in the *in vitro* MFS model. Mouse hemi-diaphragm preparations were pre-incubated with anti-GQ1b ganglioside mAb CGM3 (50 $\mu\text{g/ml}$) and subsequently treated with 40% normal human serum (NHS) with either added eculizumab (anti-human C5 neutralizing mAb; 100 $\mu\text{g/ml}$) or non-specific isotype-matched control mAb (100 $\mu\text{g/ml}$), and compared with untreated tissue. **A.** Tissues treated with NHS/eculizumab showed intense C3c staining at NMJs, similar to that of NHS/control mAb-treated tissues ($p=0.12$). **B.** Membrane attack complex (MAC) staining was not observed (intensity comparable to baseline, $p=0.92$), indicating complete inhibition of terminal complement. **C.** Axonal integrity was preserved, as assessed by presence of neurofilament (NF) signal, comparable to baseline in untreated controls ($p=0.80$). **D-F.** Illustrative immunofluorescent images of NMJs in whole-mount muscle demonstrating the protective effect of eculizumab against MAC deposition (**D**), perisynaptic Schwann cell damage (stained with ethidium homodimer-1; EthD1; **E**) and terminal motor axonal damage (**F**), as compared to NHS/control mAb treatment. Postsynaptic nicotinic acetylcholine receptor (nAChR) staining was used to delineate the NMJ. Intermediate complement protein C3c (**D**) remains abundantly deposited at NMJs.

* $p<0.01$, different from untreated; # $p<0.01$, different from control mAb-treated; scale bar: 20 μm .

***In vitro* electrophysiological analysis of NMJs and visual scoring of muscle contractility**

Left and right hemi-diaphragms with their phrenic nerve attached were dissected and pinned out in a silicone rubber-lined dish in 1.5 ml Ringer's medium at room temperature. Intracellular recordings of miniature endplate potentials (MEPPs, the postsynaptic events arising from spontaneous release of single acetylcholine quanta from the presynaptic motor nerve terminal) were made at NMJs at 20-22 °C. Muscle fibres were impaled near the NMJ with a 10-20 M Ω glass micro-electrode filled with 3 M KCl, connected to a GeneClamp 500B amplifier (Axon Instruments/Molecular Devices) for amplifying and filtering (10 kHz low-pass) of the signal. Signals were digitized, stored and analyzed (off-line) using a Digidata 1322A interface, Clampex 9.2 and Clampfit 9.2 programs (all from Axon Instruments/ Molecular Devices), Mini Analysis 6.0 (Synaptosoft) and routines programmed in Matlab (The MathWorks Inc.).

The occurrence of spontaneous asynchronous fibre twitches that are induced by a high MEPP frequency resulting from CGM3/complement-mediated presynaptic damage (Plomp et al., 1999) was scored visually every 5 min (O'Hanlon et al., 2001). The tissue scored 0 for no activity, 1 for the twitching of <10 fibres, 2 for a small amount of twitching across the tissue, 3 for a moderate amount and 4 for an extensive amount. The average score during each measuring period was calculated. The final result of CGM3/complement-mediated presynaptic damage is a silencing of synaptic transmission at the NMJ. The number of "silent" NMJs (i.e. without detectable MEPPs and occurrence of phrenic nerve-stimulation evoked muscle action potentials) was expressed as percentage of the total number of NMJs sampled (8-41) in a muscle under each experimental condition.

Muscle contraction experiments

The protective efficacy of eculizumab on *in vitro* CGM3/NHS-induced loss of nerve stimulation-evoked contraction force of the hemi-diaphragm preparation was tested. We used diaphragm from GM2synthase *null*-mutant mice because earlier studies showed a somewhat higher sensitivity of muscle of this strain to CGM3/NHS-induced loss of contraction (Bullens et al., 2002). Left and right phrenic nerve-hemi-diaphragm preparations that had been pre-incubated with 200 μ g/ml CGM3 for 3 h at 32 °C were mounted in a silicone-rubber lined dish containing 2.25 ml Ringer's medium at room temperature (20-22 °C), continuously bubbled with 95% O₂ / 5% CO₂. The ribcage side of the muscle was thoroughly fixed to the bottom of the dish by multiple small pins and the central tendon was connected via a metal hook and a string to a force transducer that was connected to an amplifier and digitizing equipment, as described earlier (Kaja et al., 2007a). Supramaximal stimuli (usually ~10 V) of 100 μ s duration were delivered every 5 min for 3 s at 40 Hz from a Master-8 programmable stimulator (AMPI). Basic tension was adjusted with a vernier control to obtain maximal stimulated tetanic contraction force (usually about 10 g). Stability of the elicited contraction was monitored for 30-50 min. Subsequently, the medium was replaced by NHS (33%) to which eculizumab (3, 6, 9, 12, 25, 50 or 100 μ g/ml) had been added and admixed for ~10 min prior to addition and the effect was monitored for 100-200 min, under continuous gentle bubbling with 95% O₂ / 5% CO₂. In the control experiments without added eculizumab, 100 μ g/ml of the control mAb was added to the NHS. The amplitude of contractions was cursor-measured off-line in the Clampfit 9.0 program (Axon Instruments/Molecular devices), at 2 s after the start of the each nerve stimulation train.

Tetanic and twitch contraction forces were also measured upon *in vitro* nerve stimulation of left hemi-diaphragm muscles that were dissected from mice that had been subjected to the *in vivo* MFS protocol.

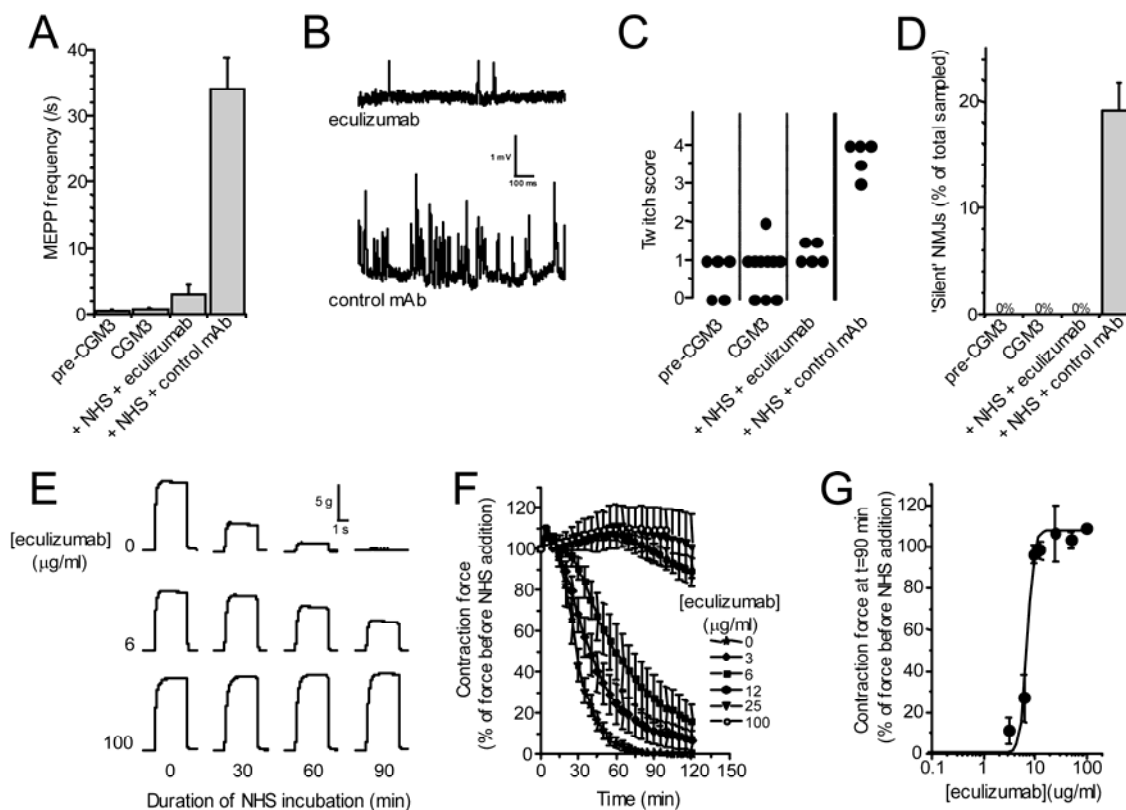


Figure 3.9. Eculizumab protects against electrophysiological and functional defects induced at NMJs in the *in vitro* MFS model. Mouse hemi-diaphragm preparations were pre-incubated with anti-GQ1b ganglioside mAb CGM3 (50 µg/ml) and subsequently treated with 40% normal human serum (NHS) with either added 100 µg/ml eculizumab (anti-human C5 neutralizing mAb) or non-specific isotype-matched control mAb. **A.** Spontaneous unquantal acetylcholine release, measured as MEPP frequency, at the NMJ. Eculizumab prevented the induction of a high MEPP frequency by NHS at CGM3-incubated NMJs ($p < 0.001$, $n = 5$ muscles). **B.** Representative 1 s duration traces obtained during incubation with NHS, either with added eculizumab (upper trace) or control mAb (lower trace). **C.** Asynchronous twitching of muscle fibres induced by NHS was largely prevented by eculizumab ($p < 0.01$, $n = 5$ muscles). **D.** Eculizumab completely prevented the occurrence of “silent” NMJs (i.e. without detectable synaptic electrophysiological signals). **E-G.** Hemi-diaphragm muscle-nerve preparations were pre-incubated with 200 µg/ml CGM3 and the protective effect of several concentrations eculizumab (0–100 µg/ml, $n = 2$ –5 muscles per concentration) added to the NHS was observed in muscle contraction force recording experiments. **E.** Examples of the contraction profiles observed at 0, 30, 60 and 90 min after the start of the NHS incubation with either no eculizumab added or 6 or 100 µg/ml added. Each contraction was elicited by supramaximal electric stimulation of the phrenic nerve for 3 s at 40 Hz. **F.** Development of the contraction loss during NHS incubation with the various concentrations eculizumab added. **G.** Concentration-effect relationship of eculizumab and the protection against loss of contraction after 90 min after the start of the NHS incubation. A Boltzmann sigmoidal curve is fitted through the data points, yielding an EC_{50} of 7.1 µg/ml. Error bars in panels **A**, **D**, **F** and **G** represent SEM.

Immunohistological analyses

Unfixed hemi-diaphragm sections were mounted in Lipshaw's M-1 mounting medium, and longitudinal cryostat sections (8-20 μm) were cut onto 3-aminopropyltriethoxysilane coated slides, air-dried, then stored at $-20\text{ }^{\circ}\text{C}$. To localize NMJs, TRITC and bodipy-labelled α -bungarotoxin (αBTx , diluted 1/750 to 1.3 $\mu\text{g}/\text{ml}$; Molecular Probes) were used. The intermediate complement component C3c was detected by incubation with FITC-labelled rabbit anti-C3c (1/300; Dako) for 1 h at $4\text{ }^{\circ}\text{C}$. MAC was detected using mouse anti-human C5b-9 (1/50; Dako) followed by FITC conjugated goat anti-mouse IgG (1/300), both for 1 h at $4\text{ }^{\circ}\text{C}$.

For NF staining, sections of unfixed tissue were pre-incubated for 1 h at $4\text{ }^{\circ}\text{C}$ with TRITC-conjugated αBTx , rinsed, immersed in ethanol at $-20\text{ }^{\circ}\text{C}$ for 20 min, then incubated overnight at room temperature with the rabbit polyclonal serum 1211 (1/750; reactive with phosphorylated NF; Affiniti Research Products Ltd.) followed by FITC conjugated goat anti-rabbit IgG (1/300; Southern Biotechnology Associates) for 3 h at $4\text{ }^{\circ}\text{C}$. All detection antibodies were diluted in phosphate-buffered saline (PBS).

pSC viability was assessed using ethidium homodimer-1 (EthD-1), a membrane impermeant dye that labels with red fluorescence the nucleic acids of membrane-permeabilized cells (Molecular Probes). In brief, nerve-muscle preparations were exposed to Ringer's containing 2 μM EthD-1. The tissue was incubated in the dark at room temperature for 1 h, rinsed in Ringer's solution and frozen for immunohistology. NMJs were identified in 15 μm cryostat sections by staining with bodipy conjugated αBTx (1.3 $\mu\text{g}/\text{ml}$), and the percentage of NMJ with EthD-1 positive nuclei at NMJs was calculated.

For illustrations, whole mount triangularis sterni muscles were incubated with CGM3 (50 $\mu\text{g}/\text{ml}$) and fluorochrome-conjugated αBTx (TRITC/FITC/Cy5) (2 $\mu\text{g}/\text{ml}$; 1:500), followed by NHS plus eculizumab or control mAb. Preparations were incubated with fluorescently conjugated αBTx and various combination of the following: mouse anti-C5b-9 (1:40; Dako), anti-C3c-FITC (1:200; Dako), or EthD-1 (2 μM) in Ringer's medium for 1 h at room temperature, rinsed in Ringer's medium, followed by fixation for 20 min in 4% formaldehyde in PBS. Unreactive aldehyde groups were quenched, by incubating with 0.1 M glycine for 10 min. The antibodies were then reapplied in PBS and agitated overnight at room temperature. For staining of intracellular antigens, muscle was fixed in 4% formaldehyde for 20 min followed by 10 min in 0.1M glycine and then incubated in a permeabilizing solution of 0.5% Triton-X100 in PBS for 30 min at RT, and either rabbit anti-S100 (1:150; Dako) or rabbit anti-NF (1:150; Chemicon) diluted in permeabilizing solution applied overnight at room temperature. Tissue was rinsed in PBS and where necessary incubated in the following fluorescently conjugated antibodies diluted 1:300; anti-rabbit IgG-FITC, anti-mouse IgG-FITC or -TRITC and agitated for 7h at room temperature in the dark. Tissue was rinsed in PBS and mounted in Citifluor mounting medium (Citifluor Products).

Image acquisition, quantitation and statistical analysis

Digital images were captured using both Zeiss Pascal confocal laser scanning microscope and Zeiss Axio Imager Z1 with ApoTome. Image-analysis measurements were made using Scion Image (Scion Corporation Frederick) image analysis software. For quantitative analysis of IgM, C3c, MAC and NF, three staining runs of each marker were performed on tissue from at least three individual hemi-diaphragms, and quantified as previously described (O'Hanlon et al., 2001). All studies were observer blinded. For immunohistological analysis of non-parametric data, statistical

comparisons were made using Mann-Whitney Test employing a 1% level of significance. For comparison of EthD-1 positive pSC at the NMJ, chi-squared test was used at 1% level of significance.

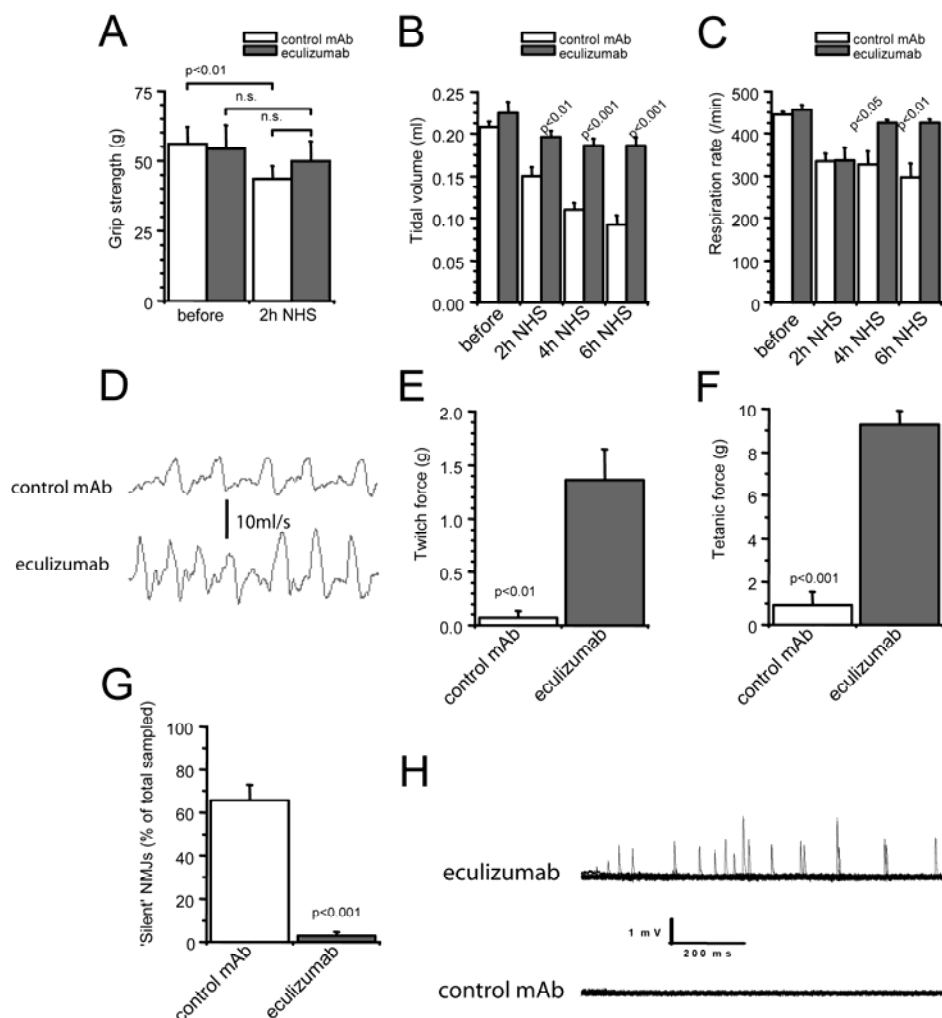


Figure 3.10. Respiratory paralysis in the *in vivo* MFS model is prevented by eculizumab due to inhibition of presynaptic block of neuromuscular transmission in the diaphragm. Mice ($n=6$) were intraperitoneally injected with 1.5 mg anti-GQ1b ganglioside mAb CGM3 and, 16 h later, with 0.5 ml 100% normal human serum (NHS) as complement source. A dose of 200 μ g eculizumab or control mAb was injected in the tail vein shortly before the NHS injection. **A.** Grip strength analysis at 2h post-NHS injection. Eculizumab prevented the loss of pulling force observed in the control mAb group ($p<0.01$). **B.** Average tidal volume and **C.** breathing rate measured with whole-body plethysmography before NHS injection and during the 2nd, 4th and 6th hour post-NHS injection. The development of breathing difficulty was prevented by eculizumab. **D.** Example 1 s traces of plethysmography flow rate obtained in eculizumab- and control mAb-treated mice. **E.** Twitch force and **F.** tetanic force elicited in the dissected diaphragm muscle by single and 40 Hz electrical nerve stimulation, respectively. Eculizumab completely prevented the severe paralysis observed in muscles from control mAb-treated mice ($p<0.01$). **G.** Eculizumab almost completely prevented the occurrence of “silent” NMJs (i.e. without detectable synaptic electrophysiological signals) in these mice ($p<0.001$). **H.** Thirty superimposed representative traces of 1 s duration obtained in NMJs of muscle from eculizumab-treated (upper traces) or control mAb-treated (lower traces) mice.

Error bars in the bar graph panels of this figure represent SEM.

n.s.: not statistically significant

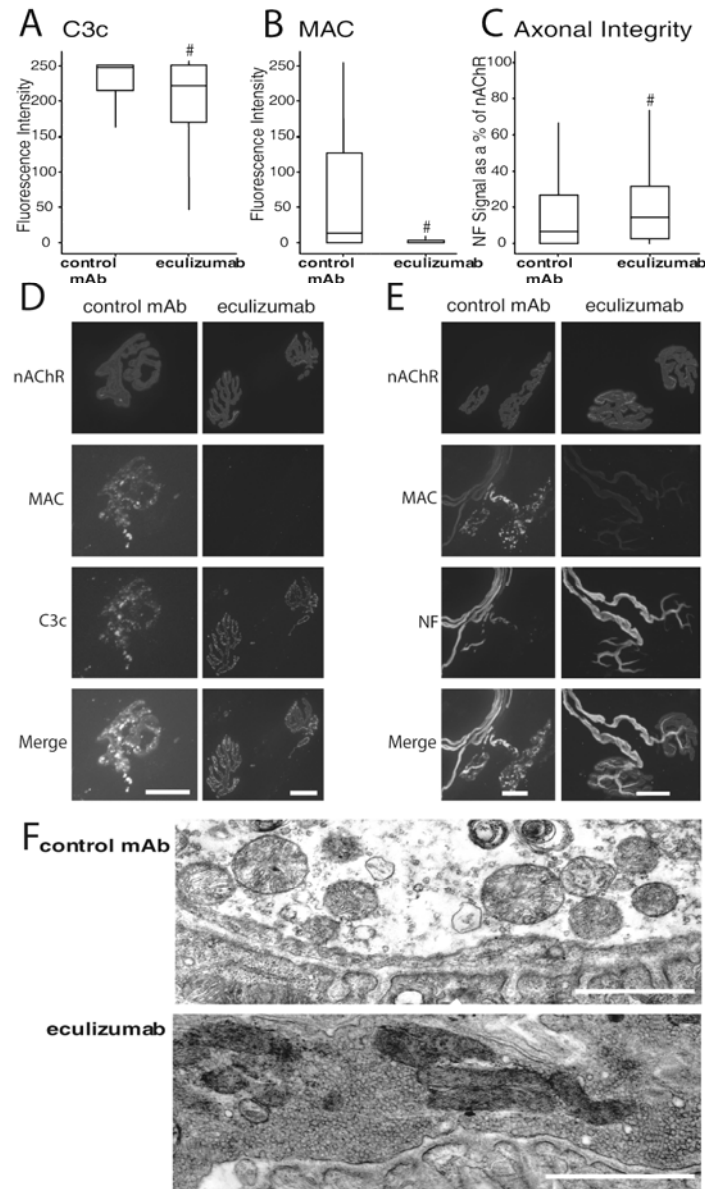


Figure 3.11. Morphological analysis of NMJs of diaphragm tissue from *in vivo* MFS mice. Mice were passively immunized with 1.5 mg anti-GQ1b ganglioside mAb, CGM3, followed 16 h later with concomitant injections of 0.5 ml 100% NHS (intraperitoneal) and eculizumab or control mAb (intravenous; 200 µg). **A-B.** C3c and membrane attack complex (MAC) deposition is reduced at neuromuscular junctions (NMJs) of eculizumab-treated mice compared with controls ($p < 0.01$). **C.** Neurofilament (NF) signal was significantly greater at NMJs of muscles from eculizumab-treated mice, compared with control ($p < 0.01$). **D-E.** Illustrative immunofluorescent images of NMJs in whole-mount muscle. Postsynaptic nicotinic acetylcholine receptors (nAChR) staining was used to delineate NMJs. **D.** C3c and MAC deposition. **E.** NF integrity and MAC deposition. **F.** Electron micrograph of eculizumab-protected nerve terminals with tightly packed synaptic vesicle and electron dense mitochondria with parallel cristae. At NMJs from control mAb-treated mice, nerve terminals are electron lucent with sparse synaptic vesicles and swollen mitochondria. # $p < 0.01$, different from control mAb-treated. Scale bars in **D** and **E**: 20 µm; and in **F**: 1 µm.

Results

Eculizumab prevents complement-mediated structural and functional lesions in the *in vitro* MFS model

In the *in vitro* MFS model, treatment of diaphragm nerve-muscle preparations with CGM3 anti-GQ1b mAb and NHS, in the presence of an irrelevant isotype matched mAb (100 µg/ml), that served as a negative control for eculizumab, induced complement activation at NMJs. This was morphologically evidenced by C3c and MAC deposition (Figure 3.8A, B, D, E and F), as well as damaged pSCs and motor nerve terminals shown by EthD-1 staining and loss of neurofilament (NF) staining, respectively (Fig. 3.8C, E and F). These features were identical to those reported before (Goodyear et al., 1999; O'Hanlon et al., 2001; Halstead et al., 2004). The addition of 100 µg/ml eculizumab to the NHS as complement source completely prevented MAC deposition (Fig. 3.8B, D, E and F; $p < 0.001$) and terminal axonal NF loss (Fig. 3.8C and F; $p < 0.001$), while the deposition of the earlier complement component C3c was not affected (Figure 3.8A and D; $p = 0.12$). Furthermore, damage to pSCs was abolished as only 2% of the 1439 NMJs investigated had one or more EthD-1 positive nuclei, compared with 33% of 1529 NMJs investigated in control mAb-treated tissue (Figure 3.8E; $p < 0.001$).

These immunohistological observations paralleled the electrophysiological and functional observations. At CGM3 pre-incubated NMJs, NHS (40%) in the presence of the control mAb induced a large increase in the frequency of MEPPs, the postsynaptic events arising from spontaneous release of single acetylcholine quanta from the presynaptic motor nerve terminal, as previously observed (Goodyear et al., 1999). This effect was almost completely prevented by 100 µg/ml eculizumab. The MEPP frequency was about 0.7 s^{-1} before and after 50 µg/ml CGM3 incubation and rose to $34.0 \pm 4.6 \text{ s}^{-1}$ in the presence of NHS with 100 µg/ml control mAb added. In contrast the MEPP frequency was only $3.1 \pm 1.3 \text{ s}^{-1}$ in the presence of NHS with 100 µg/ml eculizumab added (Figure 3.9A and B; $n=5$ muscles; $p < 0.001$).

Eculizumab did not alter the muscle fibre resting membrane potential or MEPP amplitude, rise- and decay times (data not shown). As described before (Goodyear et al., 1999; Plomp et al., 1999), asynchronous twitching of individual muscle fibre occurs during NHS incubation (median visual score 4; $n=5$ muscles). Eculizumab prevented such high grade of twitching. Only a median score of 1 was observed (Figure 3.9C; $n=5$ muscles, $p < 0.01$, Mann-Whitney test), which equals the low basal levels observed in the observation periods before and after CGM3 incubation.

The final result of anti-CGM3/complement-mediated presynaptic damage is block of synaptic transmission at the NMJ due to the inability to release acetylcholine (Goodyear et al., 1999). Eculizumab completely prevented this effect. No "silent" NMJs (i.e. absence of MEPPs and nerve-stimulation evoked muscle action potentials) were encountered, whereas in the control mAb condition $19 \pm 2.6 \%$ of the total number of NMJs sampled were observed to be silenced (Figure 3.9D; $n=5$ muscles). Furthermore, we quantified the protective effect of eculizumab on muscle paralysis in the *in vitro* MFS model. Eculizumab inhibited loss of nerve stimulation-induced contraction force of hemi-diaphragm preparations in a concentration dependent manner following 200 µg/ml CGM3 and 33% NHS treatment (Figure 3.9E and F). In the control experiments without eculizumab, NHS induced almost complete loss of contraction force within 90 min. Addition of 3 or 6 µg/ml eculizumab to the NHS slowed down the rate of contraction force loss considerably (50% loss was observed

at 39 and 60 min, respectively, compared to 27 min in the control condition; Figure 3.9F). Higher eculizumab concentrations of 9 and 12 $\mu\text{g/ml}$ were almost completely protective ($>90\%$ of the initial contraction left after 90 min) while 25, 50 and 100 $\mu\text{g/ml}$ completely prevented any CGM3/NHS-induced loss of contraction. A Boltzmann sigmoidal curve fitted through the obtained concentration-effect data points yielded an EC_{50} of 7.1 $\mu\text{g/ml}$ for eculizumab under these conditions (Figure 3.9G). These immunohistological and functional analyses show that eculizumab efficiently prevents complement-mediated pathophysiological effects in the *in vitro* MFS model.

Eculizumab protects against neuropathy and respiratory paralysis in an *in vivo* MFS model

In order to determine the *in vivo* efficacy of eculizumab, we generated an *in vivo* MFS mouse model through intraperitoneal injection of CGM3 anti-GQ1b antibody and NHS as complement source. At 16 h after the CGM3 injection, a dose of 200 μg eculizumab or control mAb was administered systemically, via the tail vein, followed by intraperitoneal injection of 0.5 ml NHS. The protocol of different injection routes of eculizumab and NHS was applied to avoid the immediate inhibition of C5 in the NHS by eculizumab within the confinement of the peritoneal cavity. Mice treated with CGM3, NHS and control mAb ($n=10$) developed a general weak appearance, in some cases a low-back posture, invaginated abdominal flanks and breathing difficulties (panting) within 2 h following the NHS injection (see supplementary material). Intravenous eculizumab injection completely prevented the development of these symptoms ($n=11$). We quantified weakness with grip strength measurement in CGM3 pretreated mice, before and 2 h after injection of control mAb or eculizumab and NHS (Figure 3.10A). Control group mice ($n=5$) pulled 56.0 ± 5.7 g just before the control mAb and NHS injection, while the eculizumab group mice ($n=5$) pulled 54.3 ± 8.4 g ($p=0.87$) at this stage. The pulling force 2 h after control mAb/NHS injection was 22% lower (43.7 ± 4.5 g; $p<0.005$). Eculizumab prevented such an effect (8% reduction; $p=0.07$). Respiratory disturbance was assessed with whole-body plethysmography continuously after mAb/NHS injection for a period of 6 h (Figure 3.10B and C; $n=5$ mice per group). The tidal volume before CGM3 injection was similar in both groups (0.21 ± 0.01 and 0.22 ± 0.03 ml in the control mAb and eculizumab group, respectively). A reduction of $\sim 50\%$ ($p<0.001$) was observed in the control mAb group at 4 and 6 h after NHS injection. Such a decrease was largely prevented by eculizumab (only 17% reduction at these time points, Figure 3.10B). Similarly, respiration rate was depressed in the control mAb group by $\sim 30\%$ ($p<0.01$) at 4 and 6 h post-NHS injection. In the eculizumab group, this reduction was only 7% (Figure 3.10C). Both treatment groups showed an equal initial reduction of about 40% of the respiration rate when measured 2 h post mAb/NHS, compared to the rate before CGM3 injection, which apparently was due to the intraperitoneal injection regimen. Example traces of the respiration flow rate obtained 4 h post-NHS are shown in Figure 3.10D.

We dissected hemi-diaphragm phrenic nerve preparations from *in vivo* CGM3/NHS treated mice at 4 h following NHS injection and performed electrophysiological measurements at NMJs, muscle contraction experiments, and detailed immunohistological analyses. Visual inspection indicated that only a very small part of the muscles from control mAb-treated mice contracted upon supramaximal electrical stimulation of the phrenic nerve, as compared to a complete contraction of muscles obtained from eculizumab-treated mice. In *in vitro* contraction experiments in

tissues harvested from passive immunizations, this effect was quantified (Figure 3.10E and F). Twitch and tetanic (40 Hz) tension was 0.07 ± 0.06 and 0.92 ± 0.61 g, respectively, in the control mAb group ($n=4$), while that in the eculizumab group ($n=4$) was 1.36 ± 0.29 and 9.26 ± 0.65 g, respectively, equalling the values of muscles from non-NHS treated age-matched mice (data not shown). With electrophysiological analysis we tested whether paralysis was caused by NMJ dysfunction. In dissected muscles from the control mAb group $66 \pm 7\%$ of the sampled NMJs had been “silenced”, i.e. there were no detectable MEPPs and no muscle action potentials upon nerve stimulations. In the eculizumab group only $3 \pm 2\%$ had been “silenced” (Figure 3.10G and H; $p<0.001$). Immunohistological analysis showed that CGM3 was deposited equally at NMJs of the two treatment groups (data not shown). C3c deposits were identified at NMJs of both groups, albeit with a somewhat less intensity in the eculizumab group (Figure 3.11A and D; $p<0.001$). MAC was clearly deposited at NMJs of muscles from control mAb-treated mice but virtually absent in the eculizumab-treated group (Figure 3.11B, D and E). Eculizumab prevented loss of terminal integrity at NMJs of CGM3- and NHS-treated mice, as evidenced by the NF signal intensity and pattern overlying the endplate region, i.e. terminal axonal branching clearly remaining intact (Figure 3.11C and E). In addition, electron microscopy showed the presence of a well-preserved presynaptic ultrastructure at the NMJ of eculizumab-treated mice in comparison with that of control mAb-treated mice which showed characteristic terminal swelling, synaptic vesicle depletion and swollen mitochondria (Figure 3.11G) (O'Hanlon et al., 2001). These electro-physiological, functional and immunohistological data show that complement-mediated terminal motor neuropathy occurs in our *in vivo* MFS model and that diaphragm paralysis contributes to the observed respiratory deficits. Most importantly, *in vivo* eculizumab treatment effectively prevented these *in vivo* neuropathological defects.

Discussion

Our previous *in vitro* studies demonstrated a key role for MAC, the pore-forming complement complex, consisting of C5b, C6, C7, C8 and C9 (Morgan, 1989), in mediating anti-ganglioside antibody-induced terminal motor neuropathy and pSC injury. We have observed a direct correlation between the extent of complement deposition, the degree of terminal axon injury, and the functional deficits at NMJs, suggesting a direct pathogenic action of complement in this model. In studies using C6-deficient NHS as a complement source (Halstead et al., 2004) or other means of preventing MAC formation (Halstead et al., 2005a), the development of morphological and electrophysiological lesions at NMJs is substantially attenuated, identifying MAC as the key factor. Similarly, in a rabbit model of anti-GM1 antibody-mediated acute motor axonal neuropathy, MAC formation is a prominent feature of the pathological phenotype suggesting a causal relationship between MAC formation and tissue injury (Susuki et al., 2007a). Whilst the contribution of MAC in mediating axonal injury in human inflammatory neuropathies cannot be readily assessed, it is evident from autopsy studies of GBS cases that MAC deposition at glial and axonal membranes is an important immunopathological factor (Hafer-Macko et al., 1996a; Putzu et al., 2000). Specific blockade of MAC may therefore be a way of preventing the pathological effects of the terminal complement while preserving the immunoprotective role of upstream complement components. Notwithstanding a

central role for complement activation with MAC formation in our MFS mouse model, it should be recognized that anti-ganglioside antibodies might exert complement independent effects on nerve function (Ortiz et al., 2001; Santafé et al., 2005; Buchwald et al., 2007; Lehmann et al., 2007). While such effects may be of relevance in certain pathophysiological circumstances, they are not evident in this model. Furthermore, antibodies found in GBS and related disorders are of the complement-fixing type (IgG1/3 and IgM; Willison and Veitch, 1994) and will inevitably activate complement.

Eculizumab is a humanized mAb directed against the complement protein C5 (Thomas et al., 1996). By binding to C5, eculizumab prevents the cleavage by C5-convertase into C5a and C5b, which is a crucial step in the terminal complement activation leading to MAC formation. Earlier components of the complement cascade are unaffected by C5 inactivation by eculizumab, such as the anaphylatoxin C3a which exerts potent proinflammatory effects, for example by mediating cellular chemotaxis. Eculizumab has recently undergone successful clinical trials in paroxysmal nocturnal haemoglobinuria, a disorder caused by complement-mediated lysis of red blood cells due to the genetic deficiency of complement inhibitory proteins (Hillmen et al., 2006; Hill et al., 2007). In the current study, eculizumab treatment was effective at ameliorating the clinical, electrophysiological and morphological symptoms of terminal motor neuropathy in experimental mouse MFS models, using anti-GQ1b mAb and NHS as complement source. Immunohistological analyses demonstrated virtually complete blockade of MAC formation at NMJs of eculizumab-treated mice and muscle-nerve preparations while C3c deposition remained almost unchanged.

In this study we have generated a new *in vivo* mouse model for MFS, displaying a respiratory paralysis phenotype that can be quantified with whole-body plethysmography that may prove useful in experimental drug studies. In this model it is likely that the almost complete paralysis of the diaphragm muscle is underlying the respiratory difficulties. The only slightly reduced grip strength of the MFS mice suggests that the respiratory paralysis is not accompanied by major limb muscle weakness. Normal contraction and NMJ function of dissected limb muscles support this idea (unpublished data, J.J. Plomp). This regional difference in paralysis is due to limited systemic distribution of the injected human complement from the peritoneal cavity rather than limited CGM3 distribution, as NMJs of dissected limb muscles have intense deposits of CGM3 and readily develop functional and morphological lesions when exposed to NHS (unpublished data, J.J. Plomp and S.K. Halstead).

One major difference between our mouse models and human MFS/GBS is the rapid time course at which the neuropathological symptoms develop, i.e. within tens of minutes in the *in vitro* model and within 2 h in the *in vivo* model, as compared with the days to weeks of onset of neuropathic symptoms in human patients. This could be due to the difference in the rate of anti-ganglioside antibody level elevation between the models and patients, i.e. a stepwise elevation by the incubation or injection occurring over seconds to minutes in the mouse models versus a slower, gradual increase in antibody production by B-cells in the patient occurring over days to weeks. The rapid onset of tissue injury in our *in vitro* and *in vivo* models required that eculizumab was added prior to the addition of NHS or injected concomitantly with NHS in order to protect against acute MAC mediated damage. This experimental protocol does not parallel the clinical context for MFS/GBS in which initiation of treatment would always follow (partial) symptom onset. Although eculizumab may not be expected to reverse existing MAC-mediated injury in patients, prompt

treatment would prevent further MAC deposition from taking place, hopefully limiting disease symptoms and thereby positively affecting the prognosis for long-term recovery (van Koningsveld et al., 2007).

Another, more fundamental, difference between our mouse MFS models and the human condition is the necessity to use human, i.e. heterologous, complement to induce the neuropathic lesions in mouse tissues. Previously, we attempted to generate an all-mouse model by intraperitoneal injection of anti-ganglioside antibody only (Goodyear et al., 1999). While we demonstrated antibody deposits overlying presynaptic nerve terminals, only sparse complement deposits were found in some mice and no functional defects were observed, even in mice lacking the complement regulators CD59a and DAF1 (unpublished data). However, mouse complement deposition at NMJs has been observed in such mice deficient in CD59a and/or DAF1 in a model of myasthenia gravis, an anti-acetylcholine receptor antibody-mediated neuromuscular disorder (Lin et al., 2002; Morgan et al., 2006; Kaminski et al., 2006). The reason for the apparent inability of complement fixing anti-ganglioside IgM or IgG mouse mAbs to lead to MAC deposition in our MFS model is unclear. It is most likely related to variations in complement activity and regulators in laboratory mice strains, as compared with humans. The complement activities of many different species have been compared in detail (Rice, 1950). In particular, a low haemolytic capacity of mouse serum seems due to the absence of classical pathway C5 convertase activity of mouse C4 (Ebanks and Isenman, 1996). For this reason, our studies have been pursued with NHS as a source of heterologous complement. Since eculizumab is specific for human C5 and unable to inhibit mouse C5 (Thomas et al., 1996), the data described here, despite being conducted in the mouse, relate very closely to human complement activation and inhibition. These results provide a basis for undertaking clinical trials with eculizumab in MFS and other neuropathies in which antibody-mediated complement activation is thought to be pathophysiologically relevant.

Supplementary material is available at *Brain* online

CHAPTER 3.3

C5 inhibitor rEV576 protects against neural injury in an *in vitro* mouse model of Miller Fisher syndrome

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Published in Journal of the Peripheral Nervous System 13 (2008) 228 - 235

Abstract

Guillain-Barré syndrome and its clinical variants, including the anti-GQ1b ganglioside-mediated Miller Fisher syndrome, comprise the world's leading cause of acute neuromuscular paralysis. Presently no specific drug therapies exist. The complement cascade, which is activated in these patients, forms an attractive drug target. In this study we tested whether the complement C5-inhibiting recombinant protein, rEV576, was able to prevent neural injury in a previously developed *in vitro* mouse model for MFS. Mouse hemi-diaphragm preparations were treated with anti-GQ1b antibody and normal human serum as a source of complement with added rEV576 or control protein. Immunohistology in control tissue showed deposition of C3c and membrane attack complex at neuromuscular junctions (NMJs), along with terminal motor axonal neurofilament degradation as well as ethidium homodimer-2 staining showing perisynaptic Schwann cell (pSC) injury. Electrophysiological and functional analyses showed block of synaptic transmission at the NMJ after an initial period of a dramatically high level of asynchronous acetylcholine release. In tissue treated with rEV576, all these indicators of motor neuronal damage were absent, except for the presence of C3c, indicating effective inhibition of C5. These results demonstrate that rEV576 effectively prevents development of neuronal and pSC damage in experimental murine neuropathy.

Acknowledgements

This work was sponsored by grants from the Wellcome Trust (#077041/Z/05/Z to HJW) and the Prinses Beatrix Fonds (#MAR04-0213 to JJP)

Introduction

The development and use of complement inhibitors have increased considerably in recent years, driven by growing understanding of the role of complement in the pathogenesis of various diseases. These include Guillain-Barré syndrome (GBS) and its clinical variants including Miller Fisher syndrome (MFS), neuropathies that are frequently associated with serum antibodies against gangliosides. Gangliosides are sialylated glycosphingolipids that are abundant in the outer leaflet of the neuronal membrane. Activated complement components have been reported in GBS patient cerebrospinal fluid and serum samples (Hartung et al., 1987; Sanders et al., 1986; Koski et al., 1987) and deposited on peripheral nerve (Hafer-Macko et al., 1996a; Putzu et al., 2000), thereby highlighting the complement cascade as a key pathway in GBS pathogenesis.

We have previously described an *in vitro* mouse model for MFS in which phrenic nerve-hemi-diaphragm preparations are incubated with anti-GQ1b ganglioside antibody or serum and normal human serum (NHS) as a source of complement (Halstead et al., 2004; O'Hanlon et al., 2001; Plomp et al., 1999). Immunohistological, functional and electrophysiological analyses showed that presynaptic binding of anti-GQ1b antibody at the neuromuscular junction (NMJ) activates complement, culminating in the formation of the lytic membrane attack complex (MAC; C5b-9), and that the ensuing damage leads to block of neurotransmission, causing paralysis of the nerve-muscle preparation. Other experimental studies on models of MFS and GBS conducted *in vivo* have also reported dependence of the pathological procession upon complement activation (Goodfellow et al., 2005; Susuki et al., 2007a).

rEV576 is a recombinant form of a soft tick (*Ornithodoros moubata*) saliva protein which protects the tick from attack by the host's complement system (Nunn et al., 2005). It is a small (17 kDa) and stable protein with a circulating half-life of 30 h in the mouse. rEV576 binds to human and mouse complement component C5, and thereby effectively inhibits cleavage into the anaphylatoxin C5a and the factor C5b, the first component necessary for the formation of MAC. Blockade of the complement cascade at the level of MAC generation is advantageous as it prevents the destructive effects of MAC while retaining the immunoprotective role of upstream complement components (e.g. C3b opsonization and phagocytosis).

Here we examined whether rEV576 is able to attenuate neural injury in the mouse *in vitro* MFS model, as assessed by immunohistological, electrophysiological and functional assays.

Methods

Mice

Male Balb/c mice were obtained from Harlan and used at 4-6 weeks of age. All animal experiments were carried out in accordance to UK Home Office guidelines (UK PPL60/3096), Dutch law and Glasgow and Leiden University guidelines.

Antibodies, sera and protein

The immunoglobulin (Ig) M anti-ganglioside monoclonal antibody CGM3 reacts with gangliosides GQ1b, GD3 and GT1a that all share a terminal disialylgalactose

structure (Goodyear et al., 1999). CGM3 concentration was measured using quantitative ELISA (Bethyl Laboratories). NHS was taken from a single donor stock that had been freshly frozen and stored in aliquots at -70°C to preserve complement activity. Both CGM3 and NHS were dialyzed against Ringer's solution (116 mM NaCl, 4.5 mM KCl, 1 mM MgCl_2 , 2 mM CaCl_2 , 1 mM NaH_2PO_4 , 23 mM NaHCO_3 , 11 mM glucose, pH 7.4) for 24 h at 4°C . Anti-C5 recombinant protein, rEV576, control protein, bovine serum albumin (BSA), were stored at -20°C until required.

***In vitro* nerve-muscle preparations for immunohistology**

Hemi-diaphragms were dissected and prepared for *in vitro* studies in Ringer's solution at room temperature (RT; $20\text{--}22^{\circ}\text{C}$), pre-gassed with 95% O_2 / 5% CO_2 as previously reported (Plomp et al., 1999). Untreated control sections of diaphragm were removed from each muscle preparation prior to any incubations and snap-frozen on dry ice for subsequent baseline immunohistological analysis of neurofilament (NF) and activated complement proteins levels. Muscles were incubated with CGM3 (50 $\mu\text{g}/\text{ml}$) for 2 h 32°C , then 30 min at 4°C , then equilibrated for 10 min at RT. Muscle preparations were then rinsed in Ringer's, and exposed to 40% NHS, as a source of complement, plus rEV576 (100 $\mu\text{g}/\text{ml}$) or control protein BSA (100 $\mu\text{g}/\text{ml}$) for 1 h at RT. NHS and rEV576/BSA were mixed 10 min prior to its addition to the muscle preparation. Tissue was assessed for levels of C3c, MAC, NF and pSC viability using ethidium homodimer-2 (EthD-2) as described below.

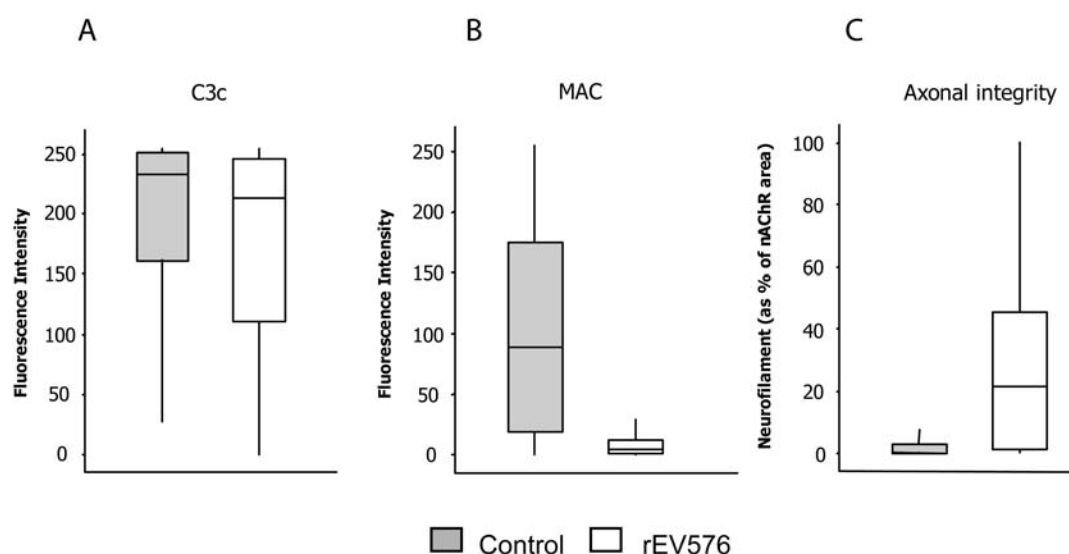


Figure 3.12. Mouse hemi-diaphragm *in vitro* preparations incubated with anti-GQ1b mAb CGM3 followed by NHS with added rEV576 or BSA, as control protein, at 100 $\mu\text{g}/\text{ml}$. The staining intensity of (A.) complement C3c was slightly reduced and of (B.) membrane attack complex (MAC) was substantially reduced over the neuromuscular junction in preparations treated with rEV576 compared with BSA ($p < 0.01$, for both C3c and MAC). C. The neurofilament signal was preserved in tissue treated with rEV576 compared with BSA-treated tissue ($p < 0.01$). nAChR: nicotinic acetylcholine receptor.

Immunohistology

Unfixed hemi-diaphragm sections were mounted in Cryo-M-Bed medium (Bright instrument company limited), and longitudinal cryostat sections (8-20 μm) were cut onto 3-aminopropyltriethoxysilane-coated slides, air-dried and then stored at -20°C . To localize the NMJ, nicotinic acetylcholine receptors (nAChR) were stained with Alexa 555-conjugated α -bungarotoxin (αBTx , diluted 1/750 to 1.3 $\mu\text{g}/\text{ml}$; Molecular Probes). Activation of the intermediate complement component C3, that leads to deposition of C3b (a complex that includes the C3c component prior to its liberation as a soluble factor, and herein termed C3c according to the antibody nomenclature) was detected by incubation with FITC labelled rabbit anti-C3c (1/300; Dako) for 1 h at 4°C . The terminal complement complex, MAC, was detected using mouse anti-human C5b-9 (1/50; Dako) followed by FITC-conjugated goat anti-mouse IgG (1/300), both for 1 h at 4°C .

For NF staining, sections of unfixed tissue were pre-incubated for 1 h at RT with Alexa 555-conjugated αBTx , rinsed, immersed in ethanol at -20°C for 20 min and then incubated overnight at RT with the rabbit polyclonal serum 1211 (1/750; reactive with phosphorylated NF; Affiniti Research Products Ltd.) followed by FITC labelled goat anti-rabbit IgG (1/300; Southern Biotechnology Associates) for 3 h at 4°C . All detection antibodies were diluted in phosphate-buffered saline (PBS).

Perisynaptic Schwann cell (pSC) viability was assessed using EthD-2, a membrane impermeant dye that labels with red fluorescence the nucleic acids of membrane-permeabilized cells (Molecular Probes). Nerve-muscle preparations were exposed to Ringer's containing 2 μM EthD-2. The tissue was incubated in the dark at RT for 1 h, rinsed in Ringer's solution and frozen for immunohistology. NMJs were identified in 15 μm cryostat sections by staining with Alexa 488-conjugated αBTx (1.3 $\mu\text{g}/\text{ml}$), and the percentage of NMJs with EthD-2-positive nuclei overlying the motor endplate was calculated.

For illustrations, muscle preparations were stained as follows. Whole-mount triangularis sterni muscles were incubated with CGM3 (50 $\mu\text{g}/\text{ml}$) and Cy5-conjugated αBTx (2 $\mu\text{g}/\text{ml}$; 1:500), followed by NHS plus rEV576 or BSA as control. Preparations were then incubated with αBTx -Cy5 (1:500) and various combination of the following primary detection antibodies, mouse anti-C5b-9 (1:40; Dako), anti-C3c-FITC (1:200; Dako) or rabbit anti-NF (1:500; clone 1211; Affiniti Research Products Ltd), in Ringer's for 1 h at RT and rinsed in Ringer's, followed by fixation for 20 min in 4% formaldehyde in PBS. Unreactive aldehyde groups were quenched, by incubating with 0.1 M glycine for 10 min. The antibodies were then reapplied in PBS and gently mixed overnight at RT. Muscle were rinsed in PBS and where necessary incubated with agitation in the following fluorescently conjugated antibodies; anti-rabbit IgG-FITC and anti-mouse IgG-tetramethyl Rhodamine iso-thiocyanate for 6 h at RT.

Image acquisition, quantitation and statistical analysis

Digital images were captured using both a Zeiss Pascal confocal laser scanning microscope and a Zeiss Axio Imager Z1 with ApoTome (Zeiss). Image analysis measurements were made using Image J (National Institutes of Health) image analysis software. For quantitative analysis of C3c, MAC and NF, staining runs of each marker were performed on non-consecutive sections of muscle from at least 5 individual hemi-diaphragms. Samples were quantified by determining fluorescence intensity (ranging from 0 (negative) to 255 (signal saturation)) of the various markers directly overlying the motor endplate region as delineated with αBTx . All studies were

observer blinded. For immunohistological analysis of non-parametric data, statistical comparisons were made using Mann-Whitney test employing a 1% level of significance. For comparison of EthD-2-positive pSC at the NMJ, chi-square test was used at 1% level of significance.

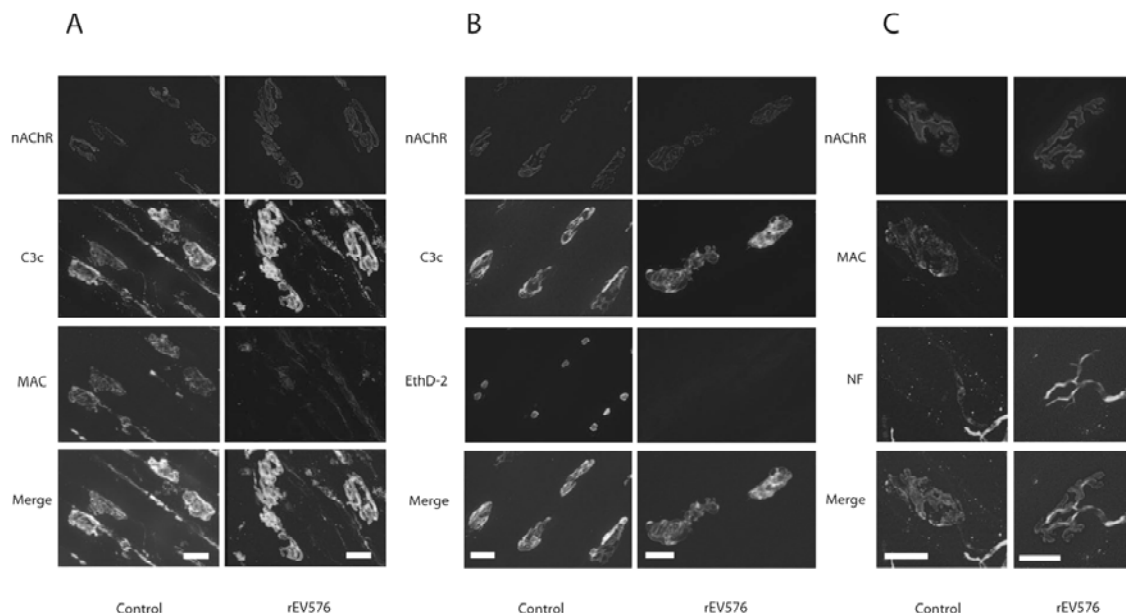


Figure 3.13. Illustrative immunofluorescent images of whole-mount muscles demonstrating neuronal and perisynaptic Schwann cell (pSC) protection at neuromuscular junctions (NMJs) by rEV576: In all panels, postsynaptic nicotinic acetylcholine receptors (nAChR) staining was used to delineate the NMJ. **A.** Intermediate complement cleavage product C3c deposits but no membrane attack complex (MAC) was seen overlying the NMJ in tissue treated with rEV576. **B.** pSC damage occurs in control-treated tissue, but is absent in tissue protected with the C5 cleavage inhibitor, rEV576. **C.** Axonal integrity is compromised in control tissue, as demonstrated by the absence of neurofilament (NF) staining overlying the NMJ. rEV576 protects against axonal damage. Scale bar: 20 μ m.

***In vitro* electrophysiology**

Hemi-diaphragms were dissected with the phrenic nerve attached and pinned out on a silicone rubber-lined dish in Ringer's medium pre-gassed with 95% O₂ / 5% CO₂ at RT. Samples were incubated for 2.5 h with 50 μ g/ml anti-GQ1b ganglioside CGM3 at 32 °C. Subsequently the preparations were rinsed in Ringer's and then treated for 1 h at RT with 33% NHS with added rEV576 or control protein BSA at 100 μ g/ml, pre-mixed 10 min before application. Using a glass microelectrode (10-20 M Ω) filled with 3 M KCl, intracellular recordings of miniature endplate potentials (MEPPs), arising from presynaptic spontaneous unquantal release of acetylcholine (ACh), were made at NMJs of preparations at RT just before the CGM3 incubation and again during the 1 h rEV576 or BSA incubation period. Recordings were amplified and filtered (10 kHz low-pass, 1 Hz high-pass) with a GeneClamp 500B amplifier and digitized and stored using a Digidata 1322A interface, Clampex 9.2 and Clampfit 9.2 (all from Axon Instruments/Molecular Devices). Data was analyzed off-line with mini-analysis 6.0 (Synaptosoft).

Visual scoring of muscle fibre twitches and evoked muscle contraction

During the electrophysiological assessment of the hemi-diaphragms incubated with NHS and rEV576/BSA, the muscles were visually scored (grades 0-4) for the occurrence of spontaneous asynchronous fibre twitching, as described before (O'Hanlon et al., 2001). These twitches are a hallmark of CGM3/complement-mediated destruction of NMJs (Plomp et al., 1999). We also scored (grades 0-2) for the extent of tetanic muscle contraction upon 40 Hz nerve stimulation. In addition, spontaneous asynchronous twitching was visually scored (0-4) in an assay using small diaphragm strips, as described before (Jacobs et al., 2002). The incubation protocol was as described above for the complete hemi-diaphragms. Scores were summed over time. After the NHS and rEV576/BSA incubation period the strips were assessed electrophysiologically for the percentage of "silent" NMJs (i.e. without any detectable synaptic electrophysiological signal), which is the final result of the presynaptic damage caused by complement.

Group comparisons were made using a Students *t*-test. A *p* value <0.05 was considered to be significant.

Results

Immunohistology

The C5 inhibitory effects of rEV576 were tested in an *in vitro* mouse model of MFS. Mouse hemi-diaphragm preparations were incubated with monoclonal anti-ganglioside antibody CGM3, followed by NHS with added rEV576 or control protein BSA.

C3c intensity at the NMJ (Figure 3.12A) was reduced by a small but statistically significant degree in hemi-diaphragm tissue treated with rEV576 (median level, 214; *n*=746) compared with control (median level, 233; *n*=727; *p*<0.01), despite similar intensities of IgM deposits (data not shown). In contrast to C3c levels and as predicted, MAC intensity (Figure 3.12B) was greatly reduced in tissue treated with rEV576 (median level, 5; *n*=678), compared to control tissue (median level, 88; *n*=704; *p*<0.01). Axonal integrity was assessed by NF staining over the NMJ (Figure 3.12C), which was abolished in control tissue (*n*=678) but preserved in rEV576-treated tissue (*n*=704). EthD-2 uptake was extensive, as expected, in tissue treated with CGM3 and NHS plus BSA control protein (54% of NMJs showed positive nuclei; *n*=2338), while only 1% of NMJs (total sampled *n*=1954) demonstrated damaged pSC in rEV576-protected tissue (chi square test, *p*<0.01).

Illustrative immunofluorescent images of whole-mount muscles demonstrating neuronal and pSC protection at NMJs by rEV576 are shown in Figure 3.13. In all upper panels, postsynaptic nAChRs were stained to delineate the NMJ, and this postsynaptic staining remains unaffected throughout, as shown in previous studies. In the presence or absence of rEV576, intermediate complement cleavage product C3c deposits were seen in tissues treated with anti-ganglioside antibody plus human complement (Figure 3.13A, B). However, no MAC was seen overlying the NMJ in tissue treated with rEV576 (Figure 3.13A, C). pSC damage as manifested by EthD-2 uptake into pSC nuclei, occurred in control-treated tissue but was absent in tissue treated with rEV576, as shown in Figure 3.13B. Axonal integrity was compromised in control tissue exposed to anti-ganglioside antibody plus NHS, as demonstrated by the

absence of NF staining overlying the NMJ, whereas rEV576 protected against this axonal damage, as shown in Figure 3.14C.

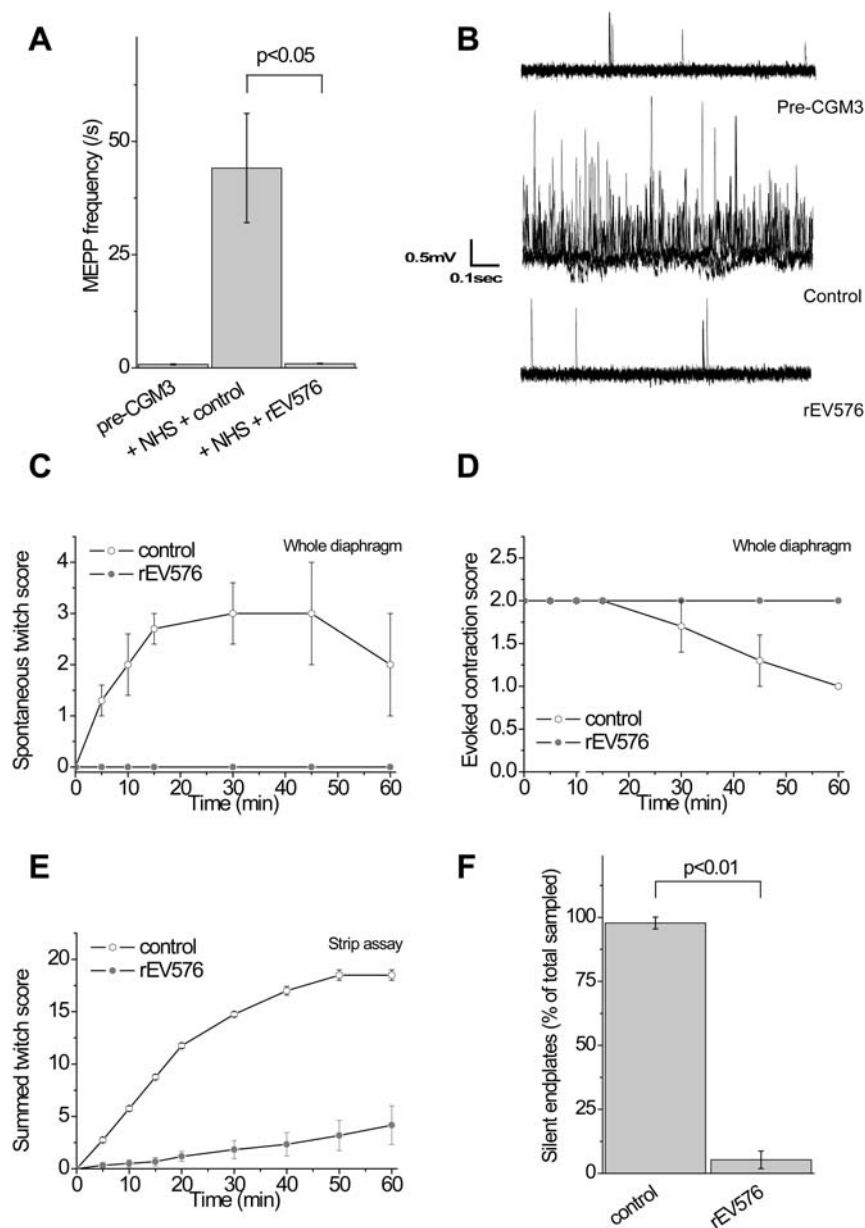


Figure 3.14. Mouse hemi-diaphragm preparations were pre-incubated with 50 $\mu\text{g/ml}$ anti-GQ1b ganglioside CGM3 for 2.5 h at 32 $^{\circ}\text{C}$. Subsequently the preparations were treated for 1 h with 33% NHS with either rEV576 or control BSA protein added at 100 $\mu\text{g/ml}$. **A.** Spontaneous unquantal acetylcholine release at the NMJ measured as MEPP frequency is significantly increased in the control group, compared to untreated NMJs. This rise was prevented in the group treated with rEV576 ($p < 0.05$; $n = 3$ muscles). **B.** Typical example traces of 1 s MEPP recordings (overlay of 5 traces). **C.** During the incubation of whole hemi-diaphragm in NHS with either rEV576 or control BSA, spontaneous twitching of the muscle fibres was scored visually. Muscle twitching was completely prevented by rEV576 ($n = 3$). **D.** During the incubation period also evoked contraction (sustained muscle contraction resulting from stimulation of the phrenic nerve) was visually scored. After 1 h rEV576 still fully protected for impairment of evoked twitching ($n = 3$). **E.** A muscle strip twitch assay was used for additional quantification of spontaneous twitching. Twitching was graded and represented as summed score over time ($n = 4-6$). rEV576 almost completely inhibited the spontaneous fibre twitches caused by NHS. **F.** rEV576 protects almost completely against the development of “silent” NMJs (i.e. without any detectable synaptic electrophysiological signal) ($p < 0.01$; $n = 4-6$).

Electrophysiology

In the *in vitro* electrophysiology study we found a MEPP frequency of $0.94 \pm 0.08 \text{ s}^{-1}$ in the muscle-nerve preparations that were pretreated with CGM3 and then incubated with NHS and rEV576. This frequency was similar to that which we measured in the untreated group (pre-CGM3; $0.77 \pm 0.10 \text{ s}^{-1}$, $p=0.24$). But in the control group that was pretreated with CGM3 and then incubated with NHS and BSA we encountered a more than 40-fold higher MEPP frequency ($44.12 \pm 12.06 \text{ s}^{-1}$, $p<0.05$). The results show that the CGM3/complement-mediated presynaptic damage which causes uncontrolled asynchronous release of acetylcholine from the presynaptic terminal is completely prevented by rEV576 ($p<0.05$; Figure 3.14A and example traces of MEPP recordings in Figure 3.14B).

Visual scoring of spontaneous twitching and nerve stimulation-evoked contraction during electrophysiological measurements also showed the protective effect of rEV576. Spontaneous twitching was absent in rEV576-treated tissue, while in the control group, it was visible from 5 min after the start of the incubation with NHS and control BSA during the whole 1 h scoring period with an average maximum score of 3 ($n=3$; Figure 3.14C). The control group also showed an impairment of evoked contraction on visually scoring, starting after 30 min of incubation. The muscles that were incubated with NHS and rEV576, however, maintained the maximal evoked muscle contraction scoring (grade 2) during the complete 1 h incubation period ($n=3$; Figure 3.14D). Similar results were obtained in the strip twitch assays. The summed twitch score at the end of the 1 h incubation was greatly different between the two groups (control 18.5 ± 0.5 , $n=4$; rEV576 4.2 ± 1.9 , $n=6$; $p<0.01$; Figure 3.14E). Afterwards we determined that almost all NMJs in the control group had been “silenced”, i.e. they were without any detectable electrophysiological signals, while rEV576 had protected muscle strips against this phenomenon (control $98 \pm 2.3\%$ and rEV576 $5 \pm 3.4\%$; $n=4$ and 6 respectively; $p<0.01$; Figure 3.14F).

Discussion

We have previously demonstrated, through the use of complement component C6 knockout mice in conjunction with C6-deficient human serum (i.e. conditions under which MAC cannot be formed) that the development of terminal motor neuropathy in our *in vitro* mouse model of MFS mediated by anti-GQ1b antibody is dependent on the generation of MAC (Halstead et al., 2004). Therefore, successful inhibition of the complement cascade with therapeutic inhibitors prior to or directed at the level of MAC within the complement cascade would be predicted to ameliorate pathological damage. In order to progress this knowledge towards clinical use in autoimmune neuropathy, we have recently studied complement inhibitors that are in preclinical or clinical development (Halstead et al., 2005a; Halstead et al., 2008a). Here we report the effects of a further inhibitor, the recombinant protein rEV576 that inhibits the conversion of human C5 into C5a and C5b and in this way prevents formation of MAC. Our data show that MAC formation is completely inhibited by rEV576 in our mouse MFS model (which involves the use of human complement), and consequently that there is complete protection of the motor nerve terminal against immune-mediated neuropathy, as assessed by electrophysiological, functional and morphological analyses. In comparison, the differential effect on C3 activation and deposition under the two conditions is minor, with a slight increase observed when

MAC is heavily deposited, most likely due to a positive feedback effect of MAC on complement activation.

The data presented here are comparable in terms of therapeutic efficacy of rEV576 to a recent study showing a substantially protective effect in an antibody and complement-mediated model of myasthenia gravis in the rat (Hepburn et al., 2007). That study also reported the known biochemical data in detail on the half-life, complement-inhibiting mechanisms and immunogenic properties of rEV576.

In our mouse model of MFS, complement is activated through the classical pathway and directed to the motor nerve terminal and pSC by membrane-bound anti-GQ1b antibody. Wallerian degeneration can result as a secondary effect after neuroimmunological axonal attack and this process has been shown to be complement dependent and delayed by complement inhibition (Ramaglia et al., 2007; Ramaglia et al., 2008). Thus, complement inhibition as a therapeutic strategy for treating peripheral nerve axonal disorders may have a dual benefit.

The field of complement therapeutics is growing rapidly with a range of agents now available for pre-clinical and clinical testing that inhibit complement activation at various stages of the cascade (Ricklin and Lambris, 2007). One particular advantage of complement inhibition at the level of C5 that rEV576 fulfils is that the antimicrobial opsonization effects of upstream complement factors are not inhibited. Conversely, this could also be a disadvantage in terms of therapeutic effectiveness when considering the possible role of macrophage and polymorphic cell infiltration in the pathogenic cascade in patients.

While this study demonstrates the therapeutic principle of axonal neuroprotection for rEV576 in a neuropathy model using an anti-GQ1b antibody directed at the motor nerve terminal, in accord with our previously published data, it is highly probable that any axonal membrane localized elsewhere and sensitized to complement attack by any other anti-ganglioside antibody would be similarly protected. Our previous work has shown complete concordance between mouse and human anti-GQ1b and other ganglioside IgG and IgM antibodies with respect to their MAC-dependent paralytic effects at the motor nerve terminal. These data should thus give further impetus towards clinical trialling of complement inhibitors in human autoimmune neuropathies.

CHAPTER 3.4

Neuropathophysiological potential of anti-ganglioside complex sera at mouse neuromuscular junctions

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To be submitted

Abstract

Some Guillain-Barré syndrome patients have serum antibodies against antigenic epitopes formed by a complex of two different gangliosides but direct evidence for pathogenicity is lacking. Here we show that anti-GM1/GD1 and anti-GM1/GQ1b complex-positive sera can induce functional damage at motor nerve terminals of mouse neuromuscular junctions *ex vivo*, similar to effects shown by us earlier of antibodies against single gangliosides. However, only about half of the 27 investigated anti-ganglioside complex sera induced neuropathogenic effects, with high variability in potency, and requiring high antigenic density. These results suggest antigenic presence of ganglioside complexes in living neuronal membranes and show pathogenic roles for some anti-ganglioside complex antibodies.

Acknowledgements

This work was sponsored by a grant from the Prinses Beatrix Fonds (#MAR04-0213, to JJP).

Introduction

With the current methods, anti-ganglioside antibodies are detected in 50-60% of patients with Guillain-Barré syndrome (GBS) (Kusunoki et al., 2008; Notturmo et al., 2009; Van Doorn et al., 2008). Gangliosides are a family of sialic acid-containing amphiphilic sphingolipids that are enriched in neuronal membranes. Anti-ganglioside antibodies are thought to be pathogenic, either by disturbing neuronal functions of gangliosides or by mediating complement activation, thereby damaging peripheral nerve axons and possibly also motor nerve terminals at the neuromuscular junction (NMJ) in some cases (Van Doorn et al., 2008; Plomp and Willison, 2009). Recently, it has been suggested that combinations of two different gangliosides can form a novel antigenic epitope and that some GBS patients have antibodies against such a complex (Kusunoki et al., 2008; Kaida et al., 2004a). Clinical correlation and fine-specificity studies estimate that 10-20% of GBS patients has anti-ganglioside complex antibodies (Notturmo et al., 2009; Kaida et al., 2007; Kuijf et al., 2007). Interestingly, antibodies against GD1a/GD1b and GD1b/GT1b complexes seem associated with disease severity (Kaida et al., 2007), although this not confirmed by others (Notturmo et al., 2009). In the GBS variant Miller Fisher syndrome (MFS), associated with anti-GQ1b ganglioside antibodies, the incidence of anti-complex antibodies seems higher: a study by Kaida and colleagues showed that 7 of 12 MFS sera contained antibodies against a complex of at least GQ1b or GT1a and another ganglioside (Kaida et al., 2006).

The observations that anti-ganglioside complex antibodies disappear upon clinical recovery (Kuijf et al., 2007) and their association with disease severity (Kaida et al., 2007) support a neuropathophysiological role. However, this has not yet been directly shown. We here performed a first investigation of 17 GBS sera with anti-GM1/GD1a complex activity and 10 GBS variant sera with anti-GM1/GQ1b complex activity for their ability to induce complement-dependent deleterious effects at mouse diaphragm motor axon terminals. Earlier, we showed that antibodies against the single ganglioside species bind to and induce structural and functional lesions at this site, electrophysiologically hallmarked by a high frequency of miniature endplate potentials (MEPPs, the postsynaptic responses to unquantal acetylcholine release), causing asynchronous muscle fibre twitches and, eventually, block of neurotransmission (Plomp and Willison, 2009).

Material and methods

Patient sera and mouse monoclonal antibodies

Acute-phase sera were obtained from 27 GBS and GBS-variant patients from Japan (# 5-15 and 18-27), The Netherlands (# 1-4) and Bangladesh (# 16-17) and stored at -80 °C. Normal human serum (NHS) from a healthy donor was stored aliquoted at -80 °C. Mouse monoclonal antibodies (mAbs) against GQ1b (CGM3; 50 µg/ml), GD1a (MOG35; 100 µg/ml) and GM1 (DG2; 100 µg/ml) were used as positive controls (Goodfellow et al., 2005; Goodyear et al., 1999). Prior to experimental use, all sera and mAbs were dialyzed overnight at 4 °C against Ringer's solution (116 mM NaCl, 4.5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 1 mM NaH₂PO₄, 23 mM NaHCO₃, 11 mM glucose, pH 7.4), pre-gassed with 95% O₂ /5% CO₂.

Enzyme-linked immunosorbent assay

Sera (1:100) were tested, as described (Kuijf et al., 2007), for IgM and IgG antibody activity against individual gangliosides and complexes, as indicated in the Results. Serum scoring an optical density (OD) of >0.2 was considered positive. For anti-ganglioside-complexes, positivity was defined as having an OD of >0.2 higher than the highest OD for an individual ganglioside (Kaida et al., 2004; Kuijf et al., 2007). All samples were tested in duplicate.

Bioassays at mouse hemi-diaphragm-phrenic nerve preparations

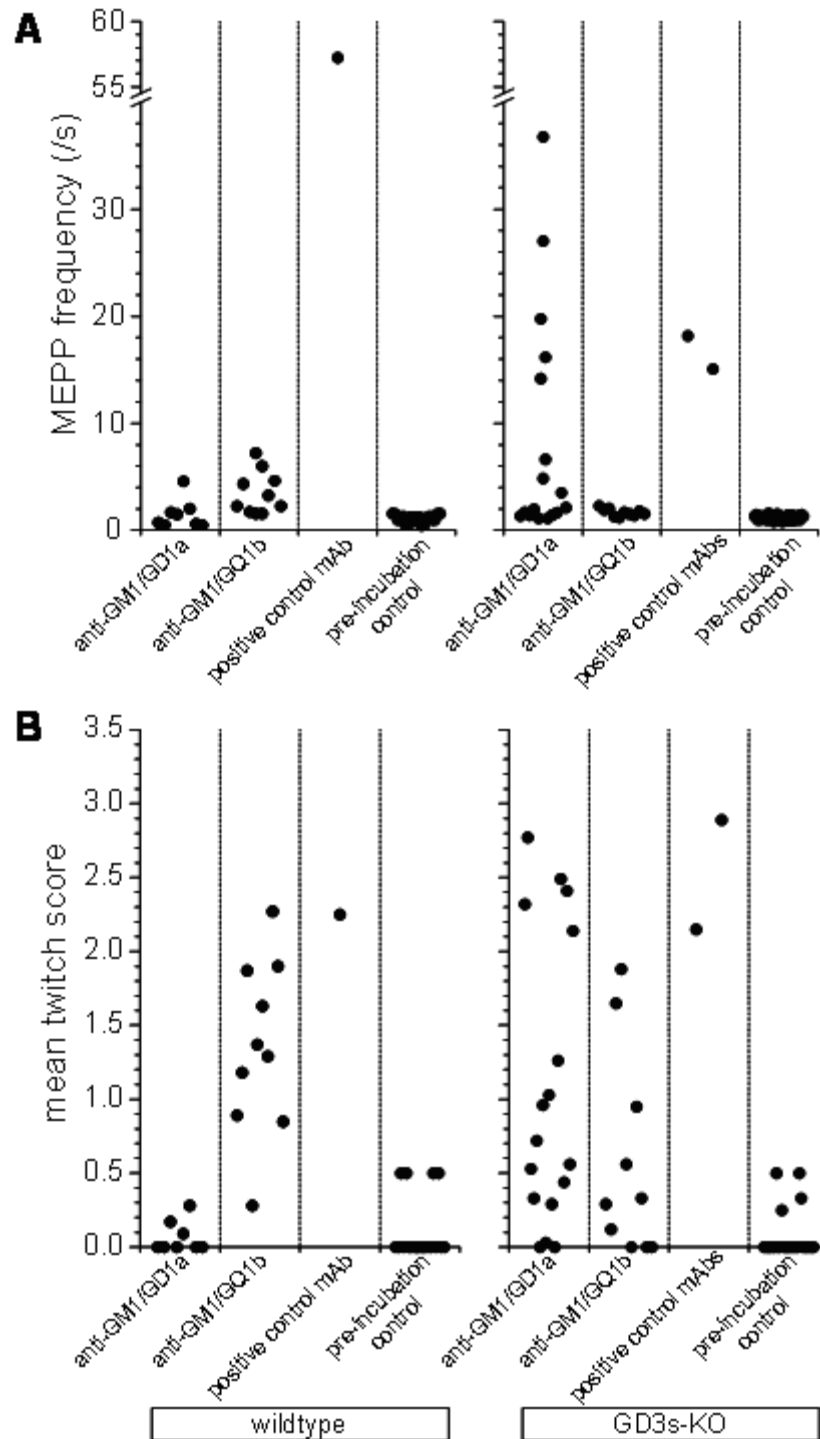
Male and female wildtype and GD3synthase knockout (GD3s-KO) mice 11 were used at 1-4.5 months of age. Hemi-diaphragms with phrenic nerves were dissected and kept in Ringer's medium at room temperature (20-22 °C). Muscles were incubated with 33% GBS heat-inactivated (30 min at 56 °C to destroy complement) serum in Ringer's medium or mouse mAbs for 3 h at 32 °C, rinsed in Ringer's medium for 10 min and exposed to 33% NHS in Ringer's medium for 1 h at room temperature. Micro-electrode recording of MEPPs (10-30 NMJs per session) and visual scoring of spontaneous asynchronous fibre twitching (0 for no twitching across the hemi-diaphragm, 1 for twitching of <10 fibres, 2 for a small amount, 3 for a moderate amount and 4 for an extensive amount) were done as described (Zitman et al., 2008; O'Hanlon et al., 2001). Depending on the available volumes, sera were tested 1 to 4 times and the mean values of the parameters were calculated. Animal experiments were carried out according to Dutch law and Leiden University guidelines.

Complement immunohistochemistry

C3 deposition at NMJs was quantified in a selection of the electrophysiologically tested samples, as described (Greenshields et al., 2009).

Results**Testing of sera for anti-ganglioside-complex specificity**

Sera were selected on the basis of their positivity for anti-ganglioside complexes (GM1/GQ1b, GM1/GD1a or GD1a/GD1b), as had been determined at their original research centre. In addition, the Japanese sera had also been tested in Japan for activity against GT1a, GD3, GalNAc-GD1a, and GA1 and ganglioside complexes GM1/GalNAc-GD1a and GD1b/GT1b. In view of possible inter-laboratory variations (Willison et al., 1999), the complete series was re-tested in Rotterdam (Erasmus MC) in a single experimental ELISA run for IgG and IgM antibodies against gangliosides GM1, GQ1b, GD1a and GD1b and ganglioside complexes GM1/GQ1b, GM1/GD1a, GM1/GD1b, GD1a/GD1b. On the basis of these ELISA studies, we classified sera into two categories (Table 3.1): 1) sera that tested positive for GM1/GD1a ganglioside complex (17 sera: # 1-17) and 2) sera that tested positive for GM1/GQ1b ganglioside complex (10 sera: # 18-27). One serum (# 26) was found negative for anti-GM1/GQ1b complex when re-tested but was still included in the second group because it had been found positive in Japan. Most sera of both categories showed additional activities against either the individual gangliosides of the complex for which they were positive, or against other individual gangliosides or other complexes (Table 3.1).

**Figure 3.15.**

A. Effect of anti-ganglioside complex sera on MEPP frequency at wildtype (left panel) and GD3s-KO (right panel) mouse diaphragms NMJs. Eight of the 17 tested anti-GM1/GD1a-complex sera induced MEPP frequency increases at GD3s-KO to a level of more than twice the pre-incubation control value. Only one serum (modestly) increased MEPP frequency at wildtype NMJs. Of the 10 investigated anti-GM1/GQ1b complex sera, 5 induced (modest) MEPP frequency rise to levels of more than twice the control value, exclusively at wildtype NMJs. **B.** Effect of anti-ganglioside complex sera on muscle fibre twitches at wildtype (left panel) and GD3s-KO (right panel) mouse NMJs. Most anti-ganglioside-complex sera that induced MEPP frequency elevation scored higher than 1 for muscle fibre twitching upon visual inspection.

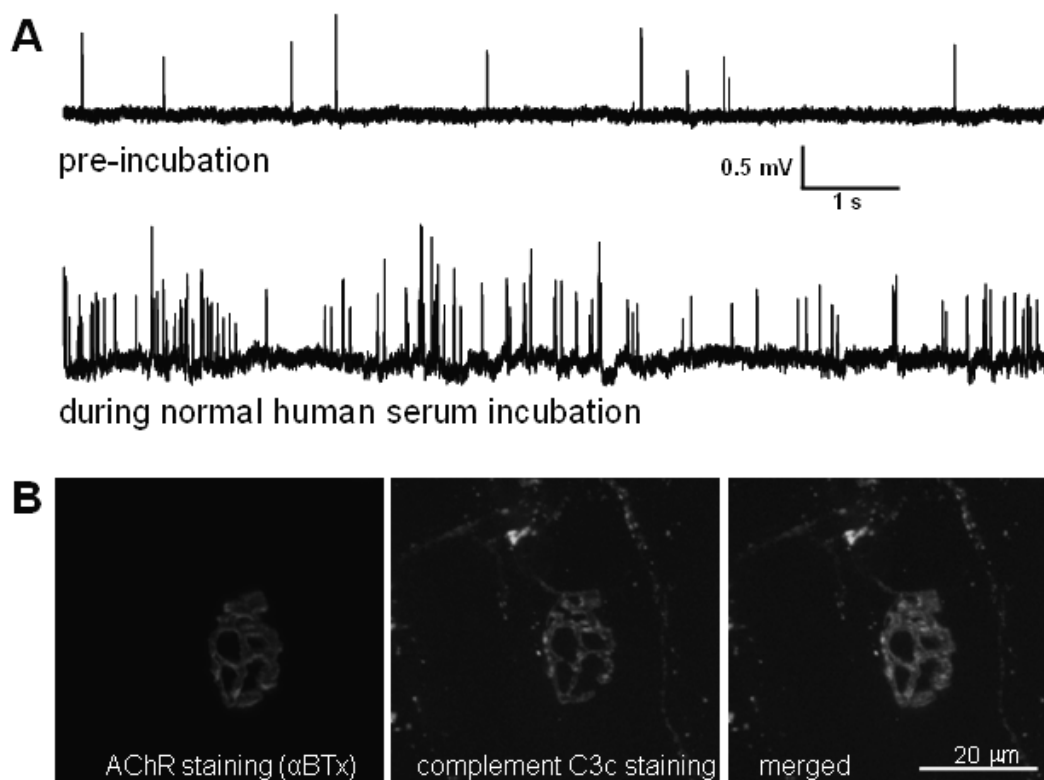


Figure 3.16.

Examples of MEPPs recorded before incubation with heated anti-GM1/GD1a-complex serum # 7 (upper panel in **A**) and during subsequent incubation with normal human serum as complement source (lower panel in **A**). Sweep length is 10 s. **B.** Example of C3c immunostaining (middle panel) at an NMJ (defined by fluorescently labelled α -bungarotoxin (α BTx) binding to acetylcholine receptors [AChR], left panel) from a muscle preparation that had been exposed to anti-GM1/GD1a complex serum # 14 and normal human serum and had shown elevated MEPP frequency and clear muscle fibre twitches.

Pathophysiological effects at mouse neuromuscular synapses

At NMJs of GD3s-KO mice (used because their membranes express upregulated density of GM1 and GD1a 15) 8 of the 17 anti-GM1/GD1a complex sera induced variable elevations of mean MEPP frequency (range 3.5-36.8 /s, the control pooled mean value before incubations was 1.2 /s; Figure 3.15 and Table 3.1) during the period of NHS incubation. From 8 of the 17 sera, sufficient material was present to also study their effects at wildtype NMJs: only one serum (# 1) showed moderate elevation of MEPP frequency (to 4.6 /s). In the anti-GM1/GQ1b series, elevated MEPPs were found for 5 of the 10 tested sera (range 3.3-7.2 /s) at wildtype NMJs. No effect was found at GD3s-KO NMJs (Figure 3.15).

Elevated MEPP frequency generally was accompanied by muscle fibre twitching, as visually scored (range 0-4) every 5 min during the 1 h NHS incubation period (Figure 3.16 and Table 3.1). Average twitching score was <0.5 in the control (pre-incubation) session. Similar scores were obtained with the GM1/GD1a series sera when tested in wildtype (range 0.0-0.3). In GD3s-KO muscles 7 of these sera scored >1.0 (Figure 3.16) with highly variable scores (range 1.0-2.8). Seven of the 10 GM1/GQ1b series

sera scored >1.0 in wildtype muscles (range 1.2-2.3). At GD3s-KO muscles, 2 of the 10 GM1/GQ1b sera scored >1.0 (1.7 and 1.9). The mean score of positive control mAbs was 2.2 or higher (Figure 3.16).

Complement deposition at the neuromuscular synapse

For the group of anti-GM1/GD1a sera, deposited amounts of complement component C3c at NMJs were found to be associated with levels of elevated MEPP frequency ($p < 0.01$, Spearman's rank correlation). In the anti-GM1/GQ1b series, NMJ C3c deposition was generally low and not consistently associated with MEPP frequency level (Table 3.1).

Discussion

The special category of anti-ganglioside complex antibodies in GBS sera was discovered by Kaida and colleagues in ELISA studies (Kaida et al., 2004a). However, no direct evidence of a pathogenic action at living neuronal membranes has been provided yet. We here report the potency of sera containing either anti-GM1/GD1a or anti-GM1/GQ1b antibodies to produce pathophysiological effects at mouse NMJs. Our earlier studies at (*ex vivo* and *in vivo*) mouse NMJs demonstrated the damaging effects of patient sera, purified antibody or mouse mAbs with (ELISA-defined) activity against either single gangliosides GQ1b, GD1a, GM1 and GD1b (for review see Plomp and Willison, 2009). These presynaptic effects were complement-dependent and electrophysiologically hallmarked by a high frequency of MEPPs, sometimes triggering a muscle fibre contraction. Roughly half of the anti-ganglioside-complex sera tested in the current *ex vivo* experiments induced such effects, albeit at rather moderate level. This indicates that anti-ganglioside complex antibodies are in principle capable of binding to live neuronal membranes and, by activating complement, can induce pathophysiological effects. Thus, these antibodies are likely of pathogenic relevance, as already suggested from the clinical association with specific patterns of paralysis (in particular cranial nerve involvement) and, in some patient groups, the requirement of mechanical ventilation (Van Doorn et al., 2008; Kaida et al., 2007). Our findings at mouse NMJs suggest that paralysis in anti-ganglioside complex antibody-positive patients may, besides axonal dysfunction, also involve some degree of NMJ synaptopathy.

However, our results are not unambiguous. First, not all anti-ganglioside complex-positive sera induced the deleterious effects at mouse NMJs. Second, the level of the effect caused by the active sera was only moderate as compared with that of the positive control mouse mAbs as well as with earlier tested patient sera with activity against single ganglioside species (Goodfellow et al., 2005; Greenshields et al., 2009; Plomp et al., 1999). These two inconsistencies may relate to variability in titre and affinity of the pathogenic antibodies amongst sera in combination with the likely existence of an antibody binding threshold for the induction of pathophysiological effects at NMJs. Third, the effects of anti-GM1/GD1a sera were only found at NMJs of GD3s-KO mice and not at those of wildtypes, indicating the requirement of high density of the targeted ganglioside complexes, possibly indicating that human patients have a special predisposing ganglioside configurations in their neuronal membranes. Fourth, many sera, especially in the GM1/GD1a series, had various additional

activities against single types of gangliosides and some sera even had additional activity against different complexes (Table 3.1). This complicates the interpretation of the result, in particular because the two monospecific anti-GM1/GD1a sera (# 16 and 17) lacked pathophysiological effects. This might suggest that besides anti-GM1/GD1a complex activity, another activity must be present in order to have enough antibody binding to exert effects. It is even not completely excluded that either antibodies against GM1a or GD1a were solely responsible for inducing effects, because all MEPP frequency-elevating anti-GM1/GD1a-positive sera were also positive for antibodies against at least one of these two individual gangliosides. On the other hand, there were also non-MEPP frequency elevating sera that contained activity against either GM1, GD1a, or both. Activity against single gangliosides was less of a problem in the GM1/GQ1b group where 2 of the 6 monospecific anti-ganglioside complex sera induced effects at wildtype NMJs and not at GD3s-KO NMJs (which contain no GQ1b; Figure 3.15), making it highly likely that the anti-ganglioside complex antibodies were responsible. However, in this series, the effects were rather modest in magnitude and complement deposition did not very well correlate with elevation of MEPP frequency, further complicating matters.

In conclusion, it is clear that a proportion of the anti-ganglioside complex sera has neuropathophysiological effects at mouse NMJ, suggesting a pathogenic role in GBS patients, but there are also some difficulties in the interpretation of these results at this stage. Development of high-affinity mouse mAbs with monospecific activity against defined ganglioside complexes is needed to allow a more detailed and extensive study of the deleterious effects of anti-ganglioside complex antibodies.

Table 3.1. (next page)

Anti-ganglioside (IgG or IgM) activity, effects on MEPP frequency (fMEPP), muscle fibre twitching and C3c deposition at neuromuscular junctions of sera with positivity for either anti-GM1/GD1a antibodies (sera # 1-17) or anti-GM1/GQ1b antibodies (sera # 18-27).

Columns anti-ganglioside activity: - : negative.

Columns fMEPP: - : < twice the control mean; + : > twice the control mean and <10 /s; ++ : between 10 and 20 /s; +++ : >20 /s; empty: not done. Elevated MEPP frequencies are also highlighted.

Columns twitching: - : <1.0; + : between 1.0 and 2.0; ++ : more than 2.0; empty: not done.

C3c staining was done in one experimental run on 17 tissue samples. Indicated is the relative intensity within this series: + : low; ++: moderate; +++ : high; empty: not done.

positive for ganglioside complex	serum#	activity against other single gangliosides or complexes				GD3s-KO mouse tissue			wildtype mouse tissue		
		GM1	GD1a	GQ1b	other	fMEPP	twitching	C3c staining	fMEPP	twitching	C3c staining
GM1/GD1a	1	-	yes	-	-	+	+		+	-	++
	2	yes	yes	-	-	-	-		-	-	+++
	3	yes	yes	-	-	-	-		-	-	
	4	yes	-	-	-	+	+		-	-	+
	5	-	-	yes	GD1b, GM1/GD1b	-	+				
	6	-	-	-	GalNAc-GD1a, GM1/GalNAc-GD1a	-	+				
	7	yes	yes	-	GD1b	++	+++	++			
	8	-	yes	yes	GD1b, GT1a	-	-	+			
	9	yes	-	-	GD1b, GA1	+	-				
	10	yes	-	-	GD1b, GM1/GD1b	-	+	+			
	11	yes	yes	-	GD1b, GalNAc-GD1a	++	+++	++			
	12	-	yes	-	GD1a/GD1b, GD1b/GT1b	+++	+++	+++	-	-	
	13	yes	-	-	GD1b, GD1a/GD1b, GD1b/GT1b	-	+		-	-	
	14	yes	yes	-	GD1b, GD1a/GD1b, GD1b/GT1b	+++	+++	+++	-	-	
	15	yes	yes	-	GD1b, GD1a/GD1b, GD1b/GT1b	++	+++	+++	-	-	
	16	-	-	-	-	-	-	+			
	17	-	-	-	-	-	-				
GM1/GQ1b	18	-	-	-	-	-	-		-	++	
	19	-	-	-	-	-	+		-	++	
	20	-	-	yes	-	-	+		+	++	
	21	yes	-	-	-	-	++		-	-	
	22	-	-	-	-	-	-	+	+	+++	++
	23	-	-	-	-	-	++		+	++	
	24	-	-	yes	GT1a	-	-	+	+	++	++
	25	-	-	-	-	-	-		-	++	
	26	-	yes	-	-	-	-	+	+	+	+
	27	-	-	-	-	-	-		-	+	

CHAPTER 3.5

The neuropathic potential of anti-GM1 autoantibodies is regulated by the local glycolipid environment in mice

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Published in the Journal of Clinical Investigation 119(3) (2009) 595 - 610

Abstract

Anti-GM1 ganglioside autoantibodies are used as diagnostic markers for motor axonal peripheral neuropathies and are believed to be the primary mediators of such diseases. However, their ability to bind and exert pathogenic effects at neuronal membranes is highly inconsistent. Using human and mouse monoclonal anti-GM1 antibodies to probe the GM1-rich motor nerve terminal membrane in mice, we here show that the antigenic oligosaccharide of GM1 in the live plasma membrane is cryptic, hidden on surface domains that become buried for a proportion of anti-GM1 antibodies, due to a masking effect of neighbouring gangliosides. The cryptic GM1 binding domain was exposed by sialidase treatment that liberated sialic acid from masking gangliosides including GD1a or by disrupting the live membrane by freezing or fixation. This cryptic behaviour was also recapitulated in solid-phase immunoassays. These data show that certain anti-GM1 antibodies exert potent complement activation-mediated neuropathogenic effects, including morphological damage at living terminal motor axons, leading to a block of synaptic transmission. This occurred only when GM1 was topologically available for antibody binding, but not when GM1 was cryptic. This revised understanding of the complexities in ganglioside membrane topology provides a mechanistic account for wide variations in the neuropathic potential of anti-GM1 antibodies.

Acknowledgements

This work was sponsored by grants from the Wellcome Trust (#077041/Z/05/Z, to HJW) and the Prinses Beatrix Fonds (#MAR04-0213 to JJP). KNG was supported by the Medical Research Council doctoral training account awarded to the University of Glasgow.

Introduction

The sialic acid-containing glycosphingolipids known as gangliosides are concentrated in plasma membrane microdomains where they modulate the topological organization and function of membrane proteins (Sandhoff and Kolter, 2003; Simons and Ikonen, 1997). Their oligosaccharide head groups protrude from the lipid bilayer into the extracellular environment to act as (co)receptors for a diverse range of glycan binding proteins including autoantibodies, sialic acid binding Ig-like lectins (siglecs), microbial toxins and viral components (Varki, 2007; Bullens et al., 2002; Willison, 2005; Crocker et al., 2007; Lencer and Tsai, 2003; Tsai et al., 2003).

In a subset of autoimmune peripheral nerve diseases, including Guillain-Barré syndrome (GBS) and multifocal motor neuropathy, autoantibody-ganglioside interactions are believed to be a critical pathogenic factor (Willison and Yuki, 2002; Yuki, 2001). Serum anti-GM1, -GD1b -GQ1b and -GD1a ganglioside antibodies are associated with nerve injury in both human clinical studies and animal models (Goodfellow et al., 2005; Kusunoki et al., 1996; Yuki et al., 2001; Plomp et al., 1999), with anti-GM1 antibodies being highly associated with motor neuropathy variants (Willison and Yuki, 2002). With respect to the antibody *induction* phase of the illness, it is clearly established that anti-GM1 antibodies can arise through molecular mimicry with structurally homologous *Campylobacter jejuni* lipooligosaccharides (LOS) (Bowes et al., 2002; Yuki et al., 1993; Yuki et al., 2004; Ang et al., 2002). In contrast, examination of the *effector* pathways through which anti-GM1 antibodies selectively bind to and induce injury in motor nerve membranes, while avoiding damage to other neural and non-neural plasma membranes containing abundant GM1, is confounded by inconsistent and often counterintuitive data (Willison and Yuki, 2002; Arasaki et al., 1993; Buchwald et al., 2007; Nores et al., 2007; Paparounas et al., 1999). In particular, the sensitivity or resistance of the membrane toward undergoing anti-GM1 antibody-mediated injury cannot be fully explained by the presence and density of plasma membrane GM1.

One reason for the uncertainties surrounding anti-GM1 effector pathways may be that protein-ganglioside interactions are typically identified by in vitro solid phase binding studies using immobilized gangliosides or structurally related natural and synthetic glycans. The translation of this in vitro binding data to physiologically and pathophysiologically relevant protein-glycan binding behaviour in intact membranes in vivo is where the complexities and inconsistencies arise. For example, an antibody that binds a specific glycan by immunoassay may apparently be unable to bind the same ganglioside when present in an intact membrane (Schwarz and Futerman, 1996). Furthermore, different anti-GM1-specific antibodies can have very different binding patterns in the CNS (Iwamoto et al., 1990; Laev and Mahadik, 1989). In addition to differences in antibody affinities, one explanation for such discrepancies might be that, within the complex environment of glycolipid-enriched microdomains, the interacting oligosaccharide headgroup is masked from the protein binding partner by surrounding molecules. Furthermore, fixation procedures might influence the antibody binding characteristics of gangliosides (Schwarz and Futerman, 1996). However, the detailed mechanisms underlying these determinants of antibody-ganglioside binding are unknown.

In the current study we addressed these issues by investigating a group of mouse and human anti-GM1 monoclonal antibodies (mAbs) for their potential neuropathogenic effects at mouse motor nerve terminals and by studying in detail the underlying

topological requirements for their binding to GM1 in neuronal membrane. Previously we showed that anti-GQ1b and anti-GD1a antibodies bind to the presynaptic motor nerve ending and activate complement, leading to membrane attack complex (MAC, C5b-9) formation, which causes intense neurotransmitter release and ultrastructural destruction, thereby blocking synaptic transmission at the neuromuscular junction (NMJ) (Goodfellow et al., 2005; Plomp et al., 1999; Goodyear et al., 1999; Halstead et al., 2004). We here show that mouse and human anti-GM1 mAbs also bring about these destructive neuropathic effects but that antigenic GM1 in the living neuronal membrane is cryptic for a proportion of the mAbs, thereby rendering them harmless, due to a masking effect of neighbouring gangliosides including GD1a. These findings indicate that anti-GM1 antibodies are pathogenically relevant in motor neuropathy and that masking and unmasking of GM1, such as may occur in membranes undergoing dynamic changes associated with normal axonal maintenance or during axonal regeneration, might have a profound influence on antibody pathogenicity (Yang et al., 2006).

Methods

Anti-ganglioside mAbs

Mice lacking complex gangliosides (GM2s-KO) were used to generate anti-ganglioside antibodies in response to inoculation of ganglioside liposomes or ganglioside mimicking LOS (Bowes et al., 2002; Townson et al., 2007; Aspinall et al., 1994). Hybridoma cells were maintained to generate stocks of mAbs, which were affinity purified by protein G sepharose chromatography (Amersham BioSciences), desalted and stored at -70 °C. Their binding properties are shown in the Table 3.2. Human mAbs were cloned as previously described (Townson et al., 2008) and their binding properties are shown in the Table 3.2.

Mice

Male and female GD3s-KO (Okada et al., 2002), GM2s-KO (Takamiya et al., 1996) and wildtype (WT) mice were used at 4-8 weeks of age. All mice were killed by a rising concentration of CO₂ in accordance with UK Home Office, Dutch law and Leiden University guidelines.

Immunohistochemistry on frozen tissue sections

Topical mAb binding studies. Diaphragm was snap-frozen and cryostat sectioned at 8 µm onto APES (Sigma-Aldrich) coated slides. For staining, mAbs were applied at 20 µg/ml and CTB-FITC (Sigma-Aldrich) at 1 µg/ml diluted in phosphate-buffered saline (PBS), along with BTx-TRITC (Molecular Probes, 1.3 µg/ml). mAbs/CTB were incubated for 3.5 h at 4 °C. For secondary detection of mAbs, slides were rinsed in PBS and anti-mouse IgG-FITC (Southern Biotech, 1.3 µg/ml in PBS) was applied for 3 h at 4 °C. All slides were mounted in Citifluor (Citifluor) following a final rinse in PBS. For CTB blocking of mAbs, sections were firstly incubated in unlabelled CTB (4 µg/ml in PBS, Sigma) or PBS as a control, for 1.5 h at 4 °C, rinsed thoroughly in PBS and mAbs applied as before.

Ig, C3, MAC and neurofilament quantification. Diaphragm (previously exposed *ex vivo* to mAbs and normal human serum as complement source) was cryostat cut at 8

and 20 μm onto APES coated slides (thicker sections were designated for neurofilament staining). All solutions were made up in PBS, and incubations done at 4°C. BTx-TRITC was used at 1.3 $\mu\text{g/ml}$ throughout. To detect mAb binding, FITC subtype specific anti-mouse IgG or anti-human IgM (Southern Biotech) was applied at 3.3 $\mu\text{g/ml}$ for 3 h at 4 °C, along with BTx-TRITC. For detection of the activated complement component C3, slides were incubated in FITC-Goat anti-human C3 (Dako, 33 $\mu\text{g/ml}$) and BTx-TRITC for 2 h at 4 °C. MAC staining was performed with mouse anti-human C5b-9 (Dako, 363 $\mu\text{g/ml}$), applied to slides for 3.5 h with BTx-TRITC. After rinsing in PBS, MAC was detected with FITC goat anti-mouse IgG2a applied at 5 $\mu\text{g/ml}$ for 3.5 h. Sections for neurofilament analysis were firstly stained for 1 h in BTx-TRITC, rinsed in PBS, and placed for 20 min in freezing ethanol (-20 °C) prior to overnight application of 1211 (rabbit polyclonal, diluted 1:750, Affinity BioReagents). Slides were rinsed and incubated in FITC goat anti-rabbit IgG (3.3 $\mu\text{g/ml}$) for 3.5 h. Slides were finally rinsed and mounted in Citifluor.

Immunostaining of live tissue

For triangularis sternae neuromuscular preparations, the ribcage was removed and pinned in a Sylgard (Sigma-Aldrich) lined dish with standard Ringer's medium (119 mM NaCl, 4.5 mM KCl, 2 mM CaCl_2 , 1 mM MgSO_4 , 1 mM NaH_2PO_4 , 23 mM NaHCO_3 , 11 mM glucose, pH 7.4 pre-gassed with 95% O_2 / 5% CO_2) and triangularis sternae was exposed. Preparations were incubated in mAbs (100 $\mu\text{g/ml}$ in Ringer's) or CTB (2 $\mu\text{g/ml}$) and BTx-TRITC (2 $\mu\text{g/ml}$) for 2 h at 32 °C, followed by 30 min at 4 °C. Tissue was thoroughly rinsed in Ringer's and fixed for 20 min at room temperature in 4% PFA (Sigma-Aldrich). Tissue was rinsed again in Ringer's and incubated for 10 min in 0.1 M glycine. For detection of mAbs, tissue was incubated overnight in subtype-specific FITC anti-mouse or anti-human IgM secondary antibodies (3.3 $\mu\text{g/ml}$ in PBS). For neurofilament staining, 1211 (Affinity BioReagents) was applied overnight (rabbit polyclonal, diluted 1 in 200) in 1% Triton-X100/PBS and following 3 rinses in PBS, was detected with cy-5 anti-rabbit IgG (3.3 $\mu\text{g/ml}$ in PBS) at 4 °C for 8 h. Following a final rinse in PBS, all immunostained muscles were whole-mounted in Citifluor.

Ex vivo pathogenesis: hemi-diaphragm

Mouse hemi-diaphragms with attached phrenic nerves were dissected out and pinned under tension in Sylgard lined dishes with oxygenated Ringer's medium. The dorsal portion of each was removed and immediately snap frozen for baseline immunohistological measurements. Hemi-diaphragms were incubated in either Ringer's (as a control) or mAbs (100 $\mu\text{g/ml}$) for 2 h at 32 °C, followed by 30 min at 4 °C and 10 min at room temperature. mAb solutions were removed and retained, then preparations rinsed in Ringer's.

Normal human serum (NHS) from a single donor stock was diluted to 40% in Ringer's, and added as a source of complement. After 1 h at room temperature, NHS was rinsed off and the diaphragm tissue snap-frozen before being processed for analysis.

Ex vivo electrophysiology at the NMJ

Left and right hemi-diaphragms were dissected with their phrenic nerve attached and mounted in standard Ringer's medium (as before) at room temperature. mAbs and normal human serum were applied as described above.

Intracellular recordings of MEPPs at the NMJ were made from muscle fibres at room temperature (20-22 °C) using a glass micro-electrode (10-20 M Ω , filled with 3 M KCl) connected to a Geneclamp 500B (Axon Instruments/Molecular devices) for amplifying and filtering (1 Hz high-pass and 10 kHz low-pass) of the signal. The signal was digitized using a Digidata 1322A interface (Axon Instruments/Molecular Devices) using Mini Analysis 6.0.3 (Synaptosoft).

Electrophysiological data is presented as group mean $\pm$ SEM of the mean muscle values calculated from the mean NMJ values. At least 10 NMJs were sampled per muscle per experimental condition.

Microscopy

For quantification of immunofluorescence over the NMJ, each experimental repeat (n of 3 animals/experiments for statistical analysis) was stained in a separate staining run, and at least 120 NMJs were imaged using constant acquisition settings for each marker. Dual colour images were acquired with a Zeiss LSM 5 Pascal microscope (Carl Zeiss). All samples were analysed observer-blinded. Semi-quantification was based on the α BTx-TRITC signal to define the NMJ, and was done with Scion Image analysis software (Scion Corporation), either by analysing the percentage of immunofluorescence over the NMJ (as previously described, (O'Hanlon et al., 2001)) or analysing the intensity of fluorescence over the NMJ. For illustrative images of whole mount triangularis sternae, 3D reconstructions were generated from z-stacks using the software package Voxx-2 (<http://www.nephrology.iupui.edu/imaging/voxx>).

Membrane treatments

For ex-vivo neuromuscular preparations, 1-5 units/ml of Neuraminidase (Clostridium perfringens, Sigma) was applied to tissue for 1-1.5 h at 32 °C. The enzyme was rinsed off and tissues incubated in mAb and/or complement as already described. For CTB pre-incubation of tissue prior to neuraminidase, unconjugated CTB (4 μ g/ml) was applied to tissue for 1.5 h at 32 °C, and thoroughly rinsed off with Ringer's prior to neuraminidase treatment. For GPI-anchored protein removal, PC12 cells were incubated at 37 °C for 1 h in 2 units/ml of PI-PLC (Sigma-Aldrich). *Ex vivo* neuromuscular preparations were treated for 1.5 h at 32 °C in 3.5 units of PI-PLC. For cholesterol depletion, 5 mM methyl- μ -cyclodextrin (Sigma-Aldrich) was incubated for 45 min at 37 °C. Cellular cholesterol was measured using Infinity Cholesterol reagent (Alpha Laboratories).

GD1a/GM1 co-localization

MOG35 (IgG2b) was primary labelled using a Zenon Mouse IgG Labelling Kit (Invitrogen) according to manufacturer's instructions. Co-localization of DG2 and MOG35 was determined using the Metamorph (version 6.3.3; Molecular Devices Corp.) "correlation plot" application to compare the fluorescence in each pixel of the FITC and TRITC channels after subtracting background fluorescence.

PC12 cell staining

Cells were grown on poly-L-lysine (Sigma-Aldrich) coated coverslips, and mAbs applied (12.5 μ g/ml in PBS) for 30 min at 4 °C. Following 3 rinses in PBS, mAbs were detected with FITC anti-mouse IgG (3.3 μ g/ml) for 45 min at 4 °C. Coverslips were rinsed again, fixed for 15 min in 4% PFA, and finally rinsed before mounting in Citifluor. For "Live/Dead staining" (Molecular Probes Inc.) a mixture of Calcein green (2 μ M) and ethidium homodimer-1 (EthD-1, 2 μ M) was applied to cells in PBS

for 30 min at 37 °C. Coverslips were rinsed in PBS, mounted in PBS and immediately imaged.

Raft isolation, affinity purification and Western blotting

Approximately 100×10^6 PC12 cells were suspended in either MOG35 or control mAb EG1 (anti-GD3, GT1a and GQ1b) at 15 µg/ml for 1 h at 4 °C. Cells were washed in chilled PBS, and kept on ice for subsequent steps. Cells were resuspended in 500 µl of 25 mM MES/150 mM NaCl, pH 6.5 (MBS), 0.5% Brij-96 (Sigma-Aldrich) and protease inhibitors (Roche Applied Science). Following incubation at 4 °C for 20 min, solubilised cells were homogenised with 10 strokes of a Dounce homogenizer. Of the lysate, 0.4 ml was mixed with 80% sucrose and overlaid successively with 2.2 ml of 30% sucrose and 1.4 ml of 5% sucrose. Following overnight centrifugation at 240,000 g, fraction 1 was collected by removing 390 µl from the top of the gradient and successive fractions through to fraction 12 were collected in this way. Protease inhibitors were added immediately upon fraction collection.

Fractions 3-5 from MOG 35 incubated cells were pooled following retention of an equal aliquot from each (for Western blotting along with other fractions). This was also done for control mAb-incubated cells. Pooled samples were halved; half for affinity purification and half retained as starting material. For affinity purification, anti-mouse IgG-coated Dynabeads (Invitrogen) were incubated with the samples for 30 min at 4 °C on a rotating mixer. Tubes were exposed to a magnet and the unbound material removed and retained. Beads were rinsed 4x in PBS, Novex loading buffer (Invitrogen) added to the rinsed beads and boiled to release the bound fractions for electrophoresis alongside unbound material and starting material.

For electrophoresis, samples were reduced by addition of 25 mM dithiothreitol (DTT, Amersham) and run on Novex 16% Tricine gels (Invitrogen). Gels were blotted overnight in a transfer cell according to manufacturer's instructions (BioRad) onto nitrocellulose membranes. Membranes were blocked in 5% non-fat milk in PBS/0.01% Tween-20 (PBST) and incubated in anti-ganglioside mAbs (20 µg/ml) or 0.1 µg/ml horseradish-peroxidase conjugated CTB (HRP-CTB, Invitrogen). After rinsing, and if required, secondary antibody (HRP-conjugated anti-mouse IgG, Sigma) was added at 0.38 µg/ml in 1% non-fat milk/PBST. ECL (Amersham) was used to develop the membranes. Fractions 1-12 were also subject to Western blotting to confirm success of the raft isolation using antibodies against flotillin (BD Biosciences), SNAP 25 (Chamberlain et al., 2001) and transferrin receptor (Invitrogen), all at 10 µg/ml.

Immunoassays

Gangliosides were purchased from Sigma Chemicals or Accurate Chemical and Scientific. ELISA was performed as previously described (Willison et al., 1999). In brief, Immunolon 2HB plates (Thermo) were coated with 100 µl of ganglioside (2 µg/ml) diluted in methanol. To create ganglioside complexes, a 50:50 (v/v) mixture of the two component gangliosides in methanol was sonicated for 3 min. A volume of 100 µl of this solution, (containing 100 ng of each ganglioside) was then applied to the appropriate wells. Control wells were coated with methanol alone. The average OD reading from control wells was subtracted from all other wells to correct for background. The mAbs DG1 and DG2, used at their GM1 half maximal binding concentrations of 0.5 µg/ml and 0.4 µg/ml respectively, and the appropriate secondary antibodies (peroxidase conjugated goat anti-mouse IgG, 1/3000, Sigma-Aldrich) were

diluted in 1% BSA (bovine serum albumin). The assay was conducted at 4 °C. For the PVDF glycoarray, gangliosides and ganglioside complexes (100 µg/ml) were prepared as described for ELISA. PVDF (polyvinylidene difluoride) membrane on glass slides was spotted (100 nl per spot) with each target ganglioside or ganglioside complex in duplicate at predefined locations on each membrane using an automated TLC sampler (Camag) as previously described (Kanter et al., 2006). Membranes were blocked with 2% BSA, and then probed with the same primary (1 µg/ml) and secondary antibodies (diluted 1/30000) as for ELISA. Chemiluminescent detection was performed using ECL plus (Amersham). Digitised film images were analysed and quantified by the array analysis component of ImageQuant TL software (Amersham).

Campylobacter jejuni

Bacteria were cultured on Skirrow agar medium (E & O Laboratories) and maintained under microaerophilic conditions. For inactivation, bacteria were suspended in PBS and placed in a UV crosslinker for 30 min or suspended in 4% PFA for 25 min.

FACS analysis

For FACS analysis, anti-ganglioside mAbs were used at 10 µg/ml, CTB-FITC at 1 µg/ml and Thy-1 (CD90, AbCam) at 7.8 µg/ml in PBS. Secondary antibodies (anti-mouse IgG-FITC) were used at 3.3 µg/ml, and cells were rinsed by pelleting in PBS. FACS analysis was performed using a FACScan flow cytometer (Becton Dickinson).

mAb	Isotype	Immunogen	Half maximal binding ^A				Affinity	
			GM1	GA1	GD1b	GD1a	K _D ^B	Ligand
Mouse								
DG1	IgG2b	HS19 LOS ^C (GM1, GT1a ⁺)	2.0x10 ³	-	-	-	1.8 x10 ⁻⁶	GM1
DG2	IgG3	GM1 liposome	2.5x10 ³	6.7x10 ²	2.0x10 ²	-	3.5 x10 ⁻⁷	GA1
MOG1	IgG3	GD1b liposome	-	-	5.0x10 ³	-	1.0 x10 ⁻⁸	GD1b
MOG35	IgG2b	O:19 LOS ^C (GM1, GD1a ⁺)	-	-	-	1.0x10 ⁴	9.5 x10 ⁻⁷	GD1a
Human ^D								
SM1	IgM	Unknown	6.5x10 ³	50	-	-	Unknown	
DO1	IgM	Unknown	5.5x10 ³	4.3x10 ³	5.7x10 ³	-	Unknown	

^A Half-maximal binding values represent the reciprocal of the antibody concentration (in mg/ml) giving half maximal binding as determined by ELISA. ^B BiaCore analysis of antibody Fab fragments was used to determine the binding affinity (K_D) of the antibodies to the shown ligands (Townson et al., 2007). ^C HS19 and O:19 are the *C. jejuni* strains from which the LOS species were isolated and used to immunize GM2s-KO mice (Bowes et al., 2002). ^D Cloned from human patients with acute and chronic motor neuropathy (Paterson et al., 1995).

Table 3.2. Mouse and human mAb ganglioside binding characteristics

Statistics

The Mann-Whitney test was used for statistical analyses of non-parametric immunohistological data, otherwise, a one way analysis of variance was used to determine statistical significance, both employing a 5% level of significance. Data are represented as box and whisker plots, with the horizontal line representing the median. One quarter of the data lying above the median, and one quarter of the data lying below the median are represented by the large box, and the vertical lines on either side show the remaining quarters of the data lying above and below the interquartile ranges. Outliers have not been shown for clarity, but were included in the statistical analysis. To assess whether binding to GM1 complexes was statistically different to GM1 alone on ELISA and PVDF array, General Linear Model ANOVA was used. Dunnett's method was employed to maintain a family error rate of <0.05 for multiple comparisons to the control level. Differences between DG1 and DG2

binding to each complex were assessed by the two sample t-test. Bonferroni's correction was applied in view of the multiple comparisons being made, again to maintain a family error rate of <0.05 . The normality of each data set was verified by the Kolmogorov-Smirnov test ($p>0.15$ indicating normality).

Results

Anti-GM1 binding characteristics of mouse and human mAbs

The origin and properties of the anti-GM1 mouse mAbs DG1, DG2, anti-GD1b mAb MOG1, anti-GD1a mAb MOG35, and human anti-GM1 mAbs SM1 and DO1 (derived from motor neuropathy patients) are shown in Table 3.2. By ELISA, both DG1 and DG2 react with GM1 with very similar half maximal binding values (0.5 and 0.4 $\mu\text{g/ml}$ respectively) as previously reported (Townson et al., 2007). Screening by ELISA against a panel of gangliosides (GM1, GM2, GM3, GD1a, GD1b, GT1a, GT1b, GQ1b, GD2, GD3, GA1) has demonstrated that DG1 only binds GM1, suggesting a binding epitope unique to GM1 that includes the internal sialic acid, whereas DG2 binds GM1, GA1, and more weakly to GD1b, suggesting the epitope principally comprises the non-reducing galactose- β 1-3 *N*-acetylgalactosamine [Gal(β 1-3)GalNAc] moiety common to the 3 glycolipids. Supplemental figure 1 shows details of relevant gangliosides and epitopes. Antibody sequencing has previously determined the variable region gene usage of DG1 and DG2 and established that they contain different immunoglobulin heavy and light chain gene rearrangements and thus have distinct clonal origins (Townson et al., 2007). Human mAb SM1 binds principally to GM1, and mAb DO1 binds to the Gal(β 1-3)GalNAc moiety common to GM1, GA1 and GD1b.

Both DG1 and DG2 bind to motor nerve terminals in frozen nerve-muscle tissue

Prior to evaluating the ability of DG1 and DG2 to mediate any pathogenic effects on nerve, we first assessed the binding potency of DG1 and DG2 to NMJs in immunohistological analyses of unfixed frozen muscle-nerve preparation of diaphragm from WT, GD3synthase deficient (GD3s-KO) and GalNAc-transferase deficient (GM2s-KO) mice. The ganglioside composition of the glycosyltransferase deficient mice is shown in supplemental figure 1. These mice are used as they are powerful tools for analysing the relevance of specific ganglioside targets, with GM1 being absent in GM2s-KO mice and overrepresented, along with GD1a and other a-series gangliosides, in GD3s-KO mice as previously reported (Okada et al., 2002). Frozen muscle sections were immunostained with DG1 or DG2 or, as positive control, with cholera toxin B-subunit (CTB), a highly specific GM1 ligand (Merrit et al., 1994). Fluorescence intensity and coverage of the NMJ area (defined by the area stained with fluorescent α -bungarotoxin [α BTx], labelling acetylcholine receptors at the NMJ) was quantitated. Under these conditions, all 3 ligands bound over nerve terminals in WT and GD3s-KO tissue (Figure 3.17). The intensity of DG1 and DG2 immunostaining at NMJs was similar in GD3s-KO tissue (~ 100 arbitrary units, AU), but the level was somewhat lower for DG1 than DG2 in WT tissue. As expected, in view of the GM1 deficiency, no DG1, DG2 or CTB staining was observed at NMJs of GM2s-KO mice.

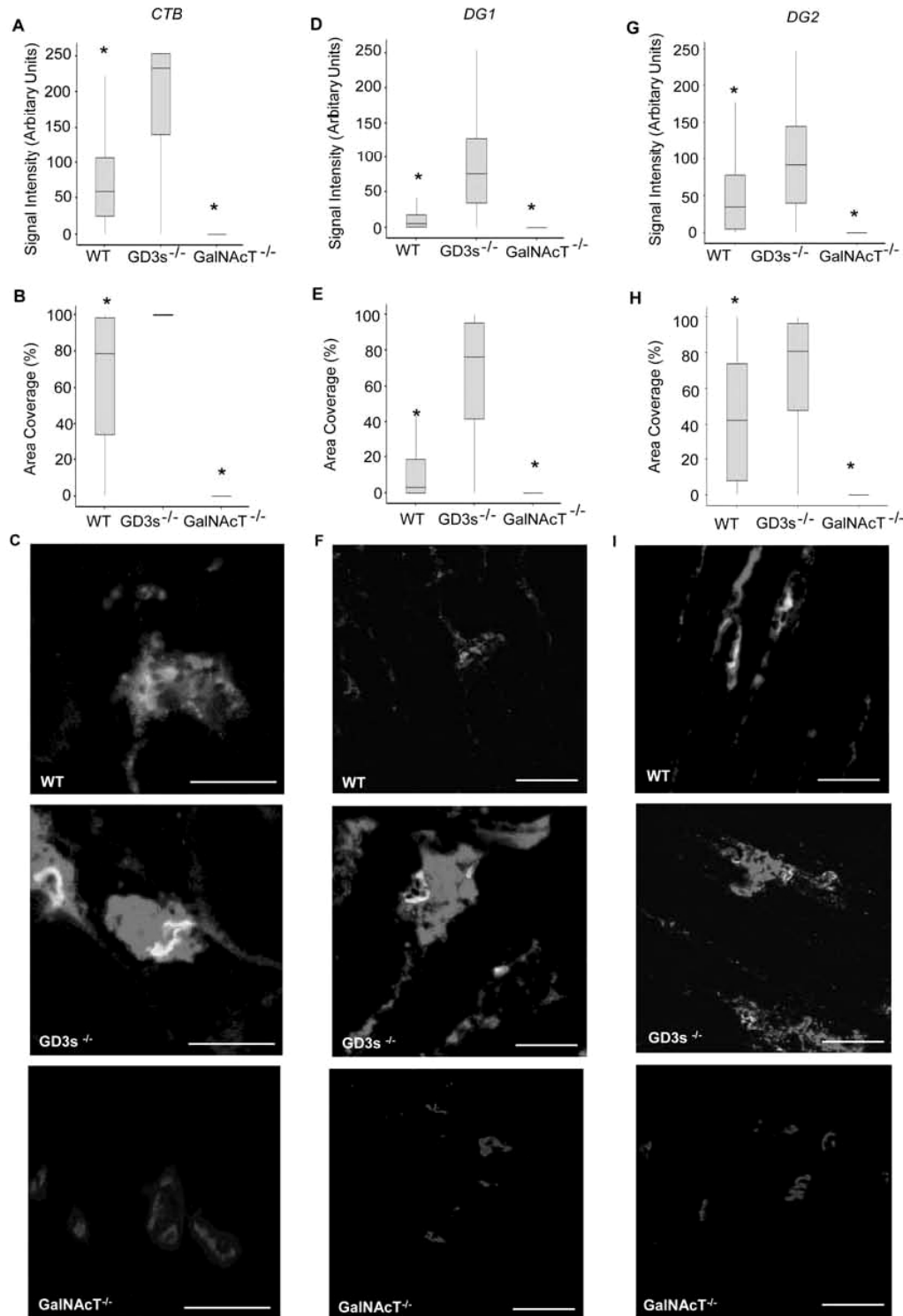


Figure 3.17. Topical immunostaining of frozen diaphragm sections from WT, GD3s-KO (GD3s^{-/-}) and GM2s-KO (GalNAcT^{-/-}) mice. Fluorescence was quantified as either the area of the NMJ (stained with α BTx-TRITC) that is covered with by anti-GM1 ligand (i.e. FITC fluorescence), or as the intensity of the FITC signal overlying the NMJ. All ligands bound most abundantly at the GD3s-KO NMJ compared to the WT NMJ. * $p < 0.05$, WT versus GD3s-KO. All images were acquired at $\times 40$ magnification. Scale bars: 60 μ m. **A. & B.** CTB fluorescence is strong at the WT and GD3s-KO, but absent at the GM2s-KO NMJ. **C.** Examples of CTB binding at NMJs of each strain, taken under constant microscope settings to represent the quantifications shown in **A** and **B**. **D. & E.** Anti-GM1 mAb DG1 stains both the WT and GD3s-KO, and is absent in the GM2s-KO NMJs. **F.** DG1 topical staining. **G. & H.** DG2 binding profile is evident at WT and GD3s-KO, but absent at the GM2s-KO NMJs. **I.** DG2 binding at the WT, GD3s-KO and GM2s-KO NMJs.

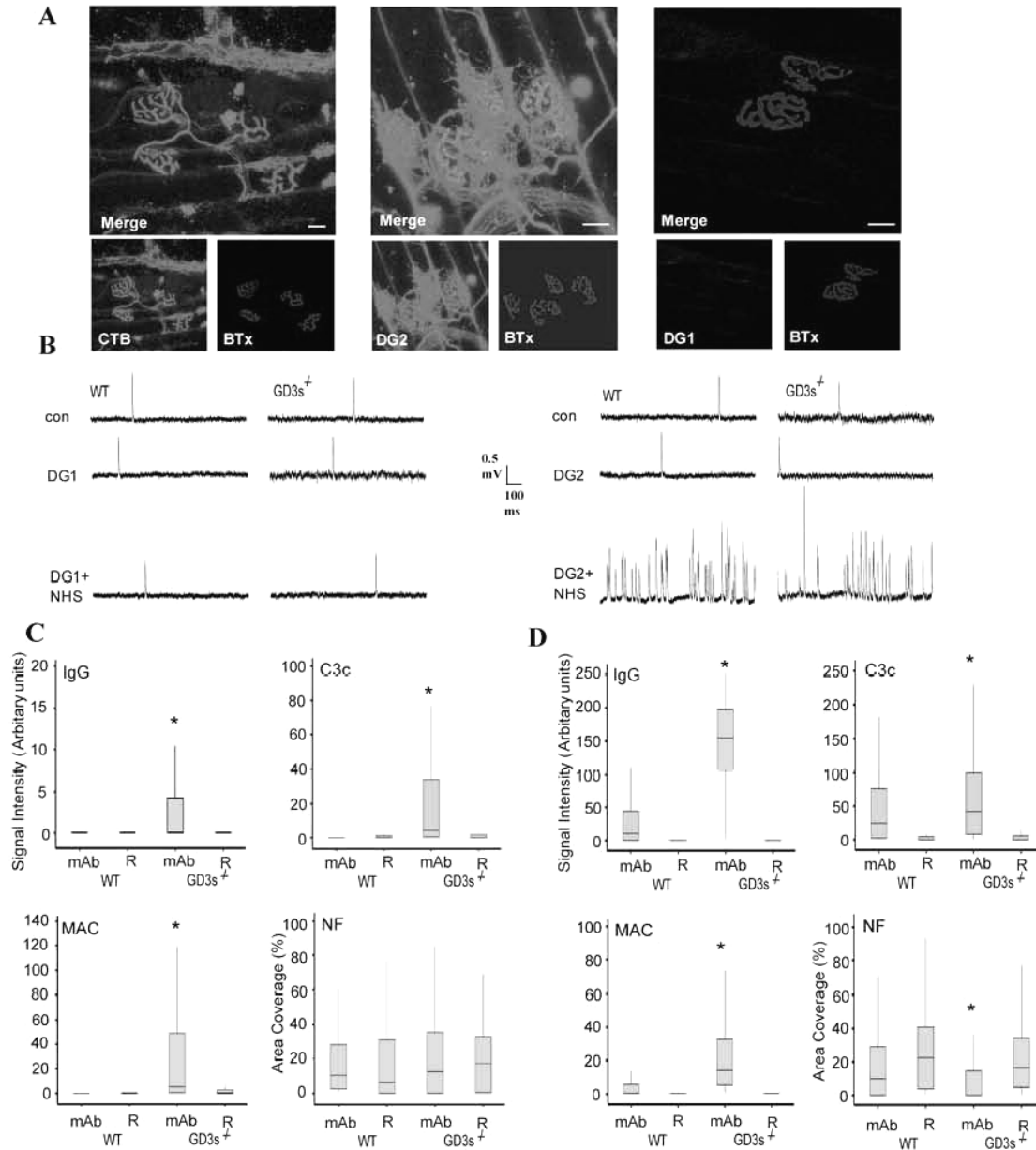


Figure 3.18. Pathophysiological effects of anti-GM1 antibodies in living nerve-muscle preparations *ex vivo*

A. Reconstructed confocal images of triangularis sterni NMJs from GD3s-KO (GD3s^{-/-}) mice, following *ex vivo* incubation in CTB, DG2 and DG1. For CTB and DG2, axonal staining is present along with staining of the parajunctional fibroblast. DG1 binding is undetectable.

B. Electrophysiology in *ex vivo* hemi-diaphragm of WT and GD3s-KO mice. mAbs were applied, followed by a source of complement, and under such conditions only DG2 caused an increase in MEPP frequency of each strain. Con, control.

C. DG1 effect in *ex vivo* hemi-diaphragm (of WT and GD3s-KO). Following *ex vivo* muscle nerve incubations in mAb (or Ringer's medium alone [R] as control), tissue was snap-frozen and sectioned for quantitative analysis. Graphs show IgG, C3c and MAC deposition (quantified as the signal intensity over the NMJ). Neurofilament (NF) loss is quantified as area coverage over the NMJ. DG1 binding is undetectable in the WT NMJ, and only weakly present in the GD3s-KO NMJ under maximal detection settings. MAC and C3c deposition do not translate into NF loss.

D. The experiment was performed as described for C., but with the mAb DG2. DG2 is detectable in WT and GD3s-KO NMJs and activates complement to cause a NF loss compared with Ringer's medium alone-incubated control tissue. For both C and D note that the Y axes have been adjusted for clarity. * $p < 0.05$ compared with the Ringer's control.

Scale bars: 20 μ m.

DG2 and DG1 have different capacities to bind to live mouse motor nerve terminals and to exert complement-mediated neuropathogenic effects

We next examined motor nerve terminal binding and possible pathophysiological effects of DG1 and DG2 at living NMJs in dissected triangularis sternae and diaphragm nerve-muscle preparations maintained as live preparations in oxygenated Ringer's medium. In positive control experiments conducted to demonstrate the presence and level of GM1 at nerve terminals, CTB bound strongly to both GD3s-KO (Figure 3.18A, left panel) and WT (not shown) nerve terminals, with strong staining of the pre-synaptic axon of the NMJ. Since GM1 is widely distributed throughout the body, albeit at low levels, staining is also seen elsewhere, including connective tissue and blood vessels; thus CTB staining over other GM1 bearing structures is also seen in the upper region of Figure 3.18A (left panel). DG2 (Figure 3.18A, middle panel) showed a similar staining pattern to CTB, with particularly pronounced staining in this image of the para-junctional fibroblast, an extralaminar cell that caps the NMJ and also bears the GM1 ligand (Court et al., 2008). GM2s-KO nerve terminals and other structures were not stained under these conditions, as expected (data not shown). DG2 strongly bound to GD3s-KO NMJs (~150 arbitrary units) and to a lesser extent (20-50 arbitrary units) to WT NMJs (Figure 3.20D). Unexpectedly, and in marked contrast to DG2 and to the situation observed in frozen sections as described above, DG1 binding was undetectable at all living WT NMJs and bound only very weakly at GM1-rich GD3s-KO NMJs (undetectable at >50% of NMJ and only at <10 arbitrary units at the most strongly positive NMJs, Figure 3.18A, C). As expected, neither of the two mAbs bound at GM2s-KO NMJs (supplemental figure 2A). Thus, whereas both DG1 and DG2 are able to bind well to GM1 at NMJs in frozen muscle-nerve preparation sections, only DG2 is capable of binding GM1 at live NMJs in physiological preparations, indicating that the topographical conformation of GM1 and/or adjacent molecules in the neuronal membrane is substantially different under these two conditions.

In order to study the ability of bound anti-GM1 antibody at live NMJs to activate complement and induce neuropathological effects, we exposed DG1- and DG2-pretreated live nerve-muscle diaphragm to normal human serum (NHS; as a complement source) and electrophysiologically monitored the occurrence of miniature endplate potentials (MEPPs, the spontaneous unquantal acetylcholine release at NMJs) with intracellular microelectrode measurement. In the presence of human serum, DG2-pretreated NMJs showed a dramatic rise in MEPP frequency in WT and GD3s-KO preparations (Figure 3.18B and supplemental figure 2B), which caused asynchronous twitching of muscle fibres which eventually subsided and was followed by complete synaptic "silence". This resulted in paralysis by block of transmission at the NMJ as judged from the inability of the diaphragm muscle to contract following phrenic nerve stimulation. As expected, in view of the finding that DG1 lacks binding activity to live WT NMJs, no such NHS-induced electrophysiological effects were observed at DG1-pretreated preparations (Figure 3.18B and supplemental figure 2C).

We screened for immunohistological evidence of antibody-mediated complement activation by assessing C3c and MAC deposition at NMJs (Figure 3.18C, D and supplemental figure 2). Both components were clearly deposited at GD3s-KO and WT nerve terminals in conjunction with DG2 binding, although with less intensity in WT. At DG1 pre-treated nerve terminals from GD3s-KO mice in which very low levels of DG1 antibody were deposited, only very low levels of C3c (median <5 arbitrary units) and MAC (median <5 arbitrary units) were present. WT NMJs pre-treated with DG1 showed no antibody binding and lacked any complement

deposition. We next examined the structural integrity of the motor nerve terminal after MAC formation at the presynaptic membrane by quantifying neurofilament staining. DG2 pre-treated NMJs from WT and GD3s-KO mice that showed MAC deposition also displayed neurofilament loss (Figure 3.20D), whereas the very low level of DG1-dependent complement activation at GD3s-KO NMJs was insufficient to induce such loss (Figure 3.18C).

Taken together, these results in living nerve-muscle preparations show that of the two anti-GM1 mAbs DG1 and DG2, only DG2 binds to motor nerve terminals and induces complement-mediated neuropathophysiological and structural effects at motor nerve terminals. Apparently, DG1 is inhibited from binding GM1, despite its abundant presence, by unknown factors in the local environment of the living neuronal membrane. The observed neuropathic effects of DG2 are identical to the complement-mediated effects we have seen previously for anti-GQ1b and anti-GD1a antibodies at NMJs (Goodfellow et al., 2005; Plomp et al., 1999; Goodyear et al., 1999; O'Hanlon et al., 2001).

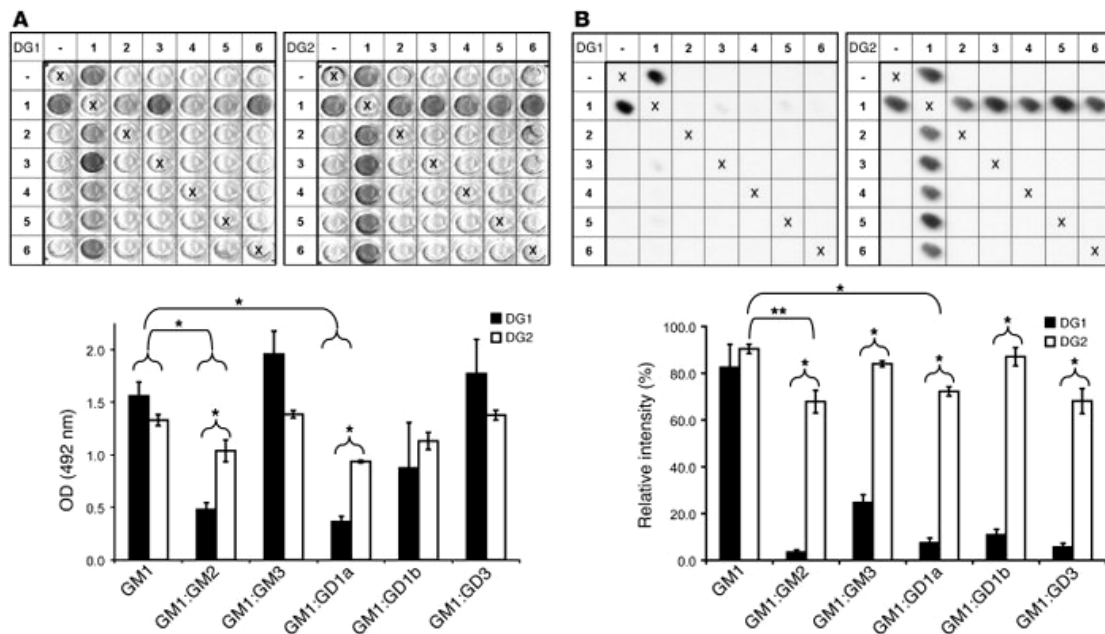


Figure 3.19. Reactivity of anti-GM1 mAbs DG1 and DG2 to ganglioside complexes containing GM1 in solid-phase

The ganglioside complex at each location is established by combining the row and column labels. Thus, coordinates 1,4 and 4,1 represent GM1/GD1a complex. Wells labelled x are negative controls (methanol only). **A.** ELISA. DG1 (left) and DG2 (right) both bind GM1 alone, with no difference in average OD. The binding of both antibodies to complexes of GM1 and GM2 or GD1a is reduced as compared with GM1 alone. No difference was observed with other combinations investigated (GM1:GM3, GM1:GD1b and GM1:GD3). DG1 binding to complexes GM1/GM2 and GM1/GD1a was less than that of DG2. Mean results $\pm$ SEM from 3 experiments are shown. **B.** PVDF glycoarrays. DG1 was the primary antibody on the left membrane, DG2 on the right. Mean results $\pm$ SEM for 3 experiments are shown. No significant difference in GM1 binding was observed for the two antibodies. DG1 binding to GM1 complexes was significantly reduced compared to GM1 alone ($p > 0.05$, significance level for these comparisons are not indicated in graph). DG2 binding GM1 complexes was marginally different compared with GM1 alone, but significant for GM1/GM2 and GM1/GD1a. The inhibitory effect of complexes on antibody binding is greater for DG1 than for DG2. The average absolute reduction in signal intensity for GM1/GD1a complex, compared to GM1 alone, is 75.1% for DG1 and 18.2% for DG2 ($p < 0.05$). Error bars indicate SEM. 1,GM1; 2,GM2; 3,GM3; 4,GD1a; 5,GD1b; 6,GD3. * $p < 0.05$, ** $p < 0.01$.

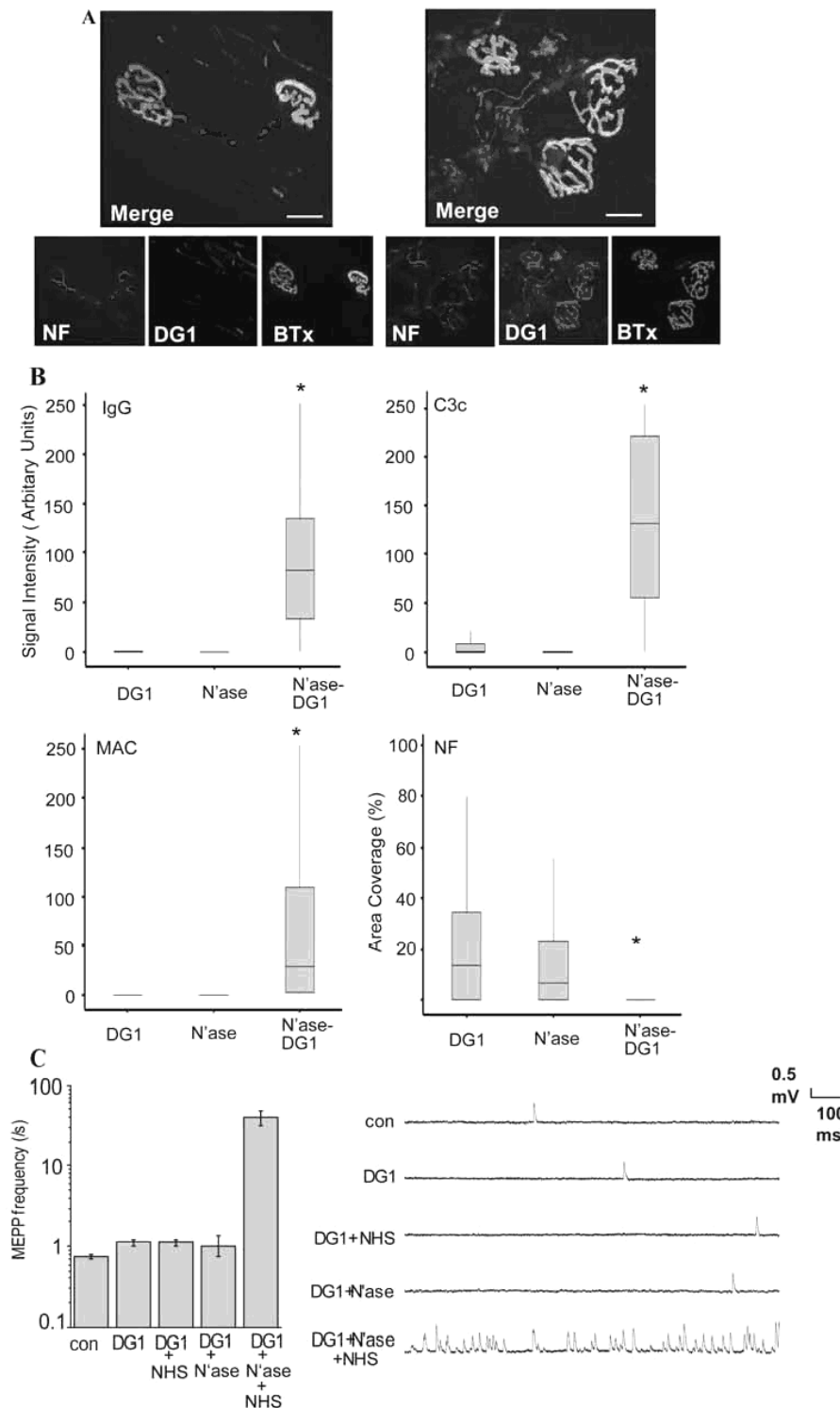


Figure 3.20. Effect of neuraminidase treatment on anti-GM1 antibody binding and pathogenic activity
A. Reconstructed confocal images of DG1 binding at *ex vivo* GD3s-KO triangularis sterni NMJs. Left: DG1 binding in living tissue is undetectable at the NMJ (stained with α BTx-TRITC). Right: DG1 binding (FITC) following neuraminidase treatment of tissue. DG1 binding overlies the NMJ, and is co-localized with the axonal NF staining (Cy5), suggesting DG1 is binding to the presynaptic aspect (the axon terminal). **B.** *Ex vivo* GD3s-KO hemi-diaphragm preparations, as described in Figure 3.180. Neuraminidase (N'ase) treatment of tissue prior to incubation with DG1 enabled DG1 to bind the NMJ (as shown by IgG deposition), fix complement, and cause a neurofilament (NF) loss. * $p < 0.05$ compared with control tissue incubated in Ringer's (minus neuraminidase) followed by DG1. **C.** Electrophysiology. DG1 and NHS as a source of complement caused a massive increase in MEPP frequency in the *ex vivo* GD3s-KO NMJ only when applied to neuraminidase-pretreated tissue. Scale bars: 20 μ m. Error bars represent SEM for experiments performed in triplicate.

The nerve terminal ligand for DG1 and DG2, and the immunopathological target for DG2 in live membranes is GM1

The absence of nerve terminal staining in GM2s-KO tissue with CTB, DG1 or DG2 (Figure 3.17) demonstrates that any potential ganglioside cross-reactivity of these GM1 ligands should only occur with the complex ganglioside series that are missing in GM2s-KO mice (supplemental figure 1). However, in order to assess for certain whether DG1 and DG2 might be binding to any of these ganglioside antigens other than GM1 at the nerve terminal in either frozen or live tissue, we conducted blocking experiments using the GM1-specific, high affinity ligand, CTB (supplemental figure 3A). In frozen tissue experiments, CTB pretreatment completely inhibited binding of DG1 and DG2 to GD3s-KO NMJs. Furthermore, CTB also inhibited DG2 binding in WT NMJs, excluding possible cross reactivity with Gal(β 1-3)GalNAc bearing epitopes. We next demonstrated that DG2 (which binds GM1, GA1 and GD1b in ELISA) does not bind GD1b in the living neuronal membrane as follows. We first showed the presence of GD1b at NMJs by incubating tissues with an anti-GD1b monospecific antibody, MOG1 (Boffey et al., 2005), and observing intense immunohistochemical staining at motor nerve terminals of WT mice (but neither GM2s-KO nor GD3s-KO mice that both lack GD1b). MOG1 also induced associated complement deposition and structural injury, as determined by neurofilament loss (supplemental figure 3B). We then showed that MOG1 binding was unaffected by CTB blockade (supplemental figure 3C). These data therefore exclude the possibility that DG2 is binding GD1b in the living tissue. They thus demonstrate that the Gal(β 1-3)GalNAc epitope on GD1b is cryptically orientated for DG2 binding *in vivo*, whereas the MOG1-reactive GD1b epitope is available for MOG1 binding *in vivo*. Since GA1 (asialo-GM1) is absent from the mammalian nervous system (Seyfried et al., 1996), this potential cross-reactive antigen is not relevant in this context and potential binding to Gal(β 1-3)GalNAc-bearing glycoproteins is excluded by the absence of staining in GM2s-KO mice. Furthermore, and consistent with the complete blocking seen in frozen tissue sections, CTB pre-incubation of *ex vivo* muscle tissue from WT mice inhibits the complement mediated actions of DG2, evidenced by abolition of C3c and MAC depositions over the NMJ (supplemental figure 3D). Collectively, these data indicate that DG2 is only binding GM1 in nerve terminal membranes *in vivo*. It should also be noted that the binding of DG2 to GD1b in ELISA is very weak, (half maximal binding, 2×10^2 , see Table 3.2), further indicating that it is unlikely to bind avidly to GD1b in membranes.

Inhibitory interactions between GM1 and adjacent gangliosides form under solid-phase conditions

We speculated that the inability of DG1 to bind GM1 in the living plasma membrane was due to a masking effect arising from *cis* interactions with adjacent gangliosides and that these were disrupted following the death of tissue and subsequent processing to frozen sections. Since GD3s-KO mice are only able to synthesise a-series gangliosides, the most abundantly expressed species being GM1 and GD1a, we concluded that the masking effect in this mouse strain was most likely due to a GM1/GD1a *cis* interaction, notwithstanding the possibility that other gangliosides (e.g. GM2, GM3) could mediate similar blocking function in membranes with other ganglioside compositions. To investigate the potential inhibitory interaction between GM1 and GD1a for DG1 binding, we first attempted to recapitulate the *in vivo* interaction in solid phase binding assays using ELISA and then using newly developed PVDF membrane formats (Figure 3.19). In ELISA, conducted with

GM1:GD1a-coated at a 1:1 ratio by weight, DG1 was significantly inhibited and DG2 partially inhibited from binding GM1 in the presence of GD1a (Figure 3.19A). Both monoclonal antibodies were used at their GM1 half maximal binding concentrations of 0.5 $\mu\text{g/ml}$ and 0.4 $\mu\text{g/ml}$ respectively. Furthermore, DG1 was significantly more impaired than DG2 from binding the GM1/GD1a complex. This inhibitory effect was not unique to GD1a, also occurring to significant extent with GM1:GM2 and a mild extent (not significant) with GM1/GD1b. In marked contrast, complexes of GM1/GM3 and GM1/GD3 did not produce any inhibitory effects on DG1 or DG2 binding, excluding the possibility that non-specific displacement of GM1 by a second, non-reactive ganglioside is accounting for the lower signal seen for the inhibitory GM1/ganglioside complexes.

When isolated GM1 was displayed on PVDF membranes as dot blots, there was no significant difference in the binding of DG1 and DG2 (Figure 3.19B). When GM1 was complexed with a range of other gangliosides, DG1 was almost completely inhibited from binding GM1. In contrast, DG2 binding to GM1 was minimally affected, although the difference in binding did reach significance for GM1/GM2 and GM1/GD1a complexes (Figure 3.19B). Throughout these PVDF studies, the reduction in complex binding for DG1 was substantially and significantly greater than for DG2. Under the PVDF array and ELISA assay conditions used in this study, DG2 is not demonstrated to bind to GD1b because of the very low half-maximal binding value for the DG2/GD1b interaction.

Inhibitory interactions between GM1 and GD1a are not present in *C. jejuni* LOS

To investigate whether or not this inhibitory interaction between GM1 and GD1a was a ganglioside-specific phenomenon or might also occur in other situations when GM1 and GD1a oligosaccharides were juxtaposed, we exploited the neuropathy-associated *Campylobacter* species that bear GM1 and GD1a mimics on their LOS (it should be noted that DG1 was cloned following immunization with LOS). A Penner O:19 *Campylobacter jejuni* isolate bearing both GM1 and GD1a mimics (50:50 ratio) in the core oligosaccharide was assessed for binding to DG1 (anti-GM1) and MOG35 (anti-GD1a) mAbs and CTB by FACS, in comparison with a non-ganglioside mimicking O:3 *C. jejuni* strain. DG1 was able to bind well to both live and killed (ultraviolet light or paraformaldehyde) O:19 isolate, indicating that an inhibitory complex between the GM1 and GD1a mimicking moieties of LOS is not present (supplemental figure 4). MOG35 and CTB also bound well to the O:19 isolate, confirming the presence of both the GM1 and GD1a mimics, and no ligands bound the control O:3 isolate (data shown for CTB only).

Access of DG1 to GM1 in the living membrane is prevented by a masking effect of GD1a

Based on the above data, we considered that the inability of DG1 to bind GM1 on the living neuronal membrane was due to closely adjacent gangliosides, including GD1a, which might *cis*-interact with GM1 to create a masking effect. Using live nerve-muscle tissue preparations from GD3s-KO mice (which highly overexpress GM1 and GD1a), we cleaved the terminal sialic acid from GD1a with neuraminidase treatment thereby converting GD1a to GM1, and subsequently assessed the live tissue for DG1 binding (Figure 3.20). This pretreatment massively promoted DG1 binding at the NMJ (Figure 3.20A, B), which subsequently activated complement, as shown by C3c and MAC deposition (Figure 3.22B). It thereby induced the previously described pathophysiological cascade (Figure 3.20C), characterized by increased MEPP

frequency, muscle fibre twitching, and paralysis, described above for DG2. This unmasking effect for DG1 was also evident in neuraminidase-treated tissue from WT C57Bl/6 and Balb/c mice (supplemental figure 5A).

In control experiments, we verified that neuraminidase treatment was able to digest most of the GD1a present at motor nerve terminals as assessed by a substantial reduction in the binding of the anti-GD1a mAb, MOG35 after treatment, in comparison with strong binding before treatment (supplemental figure 5B). DG1 binding following neuraminidase treatment was also completely blocked by pre-incubation with CTB, indicating that binding of DG1 was GM1-specific (supplemental figure 5C). Since neuraminidase treatment creates *de novo* GM1 from GD1a, it is very likely that this *de novo* GM1 would also be capable of acting as a ligand for DG1. Such binding confounds (without contradicting) the conclusion that masked “native” GM1 is released from its *cis* inhibition with GD1a by neuraminidase treatment to become available for DG1 binding. In order to investigate the extent to which the DG1 binding was directed to *de novo* GM1 generated from GD1a (rather than the pre-existing, native GM1), we first blocked native GM1 in the tissue preparation with CTB and then treated the tissue with neuraminidase, followed by probing with DG1 (supplemental figure 5D). Under these conditions DG1 binding to CTB-pretreated tissue was greatly reduced compared with binding to CTB-untreated tissue, indicating that a proportion of DG1 binding was to native GM1, unmasked from GD1a by neuraminidase treatment. After neuraminidase incubation, DG2 bound equally to both CTB pre-treated and untreated tissue, indicating that abundant *de novo* GM1 produced from GD1a was available for DG2 binding. The relatively weak binding of DG1 in comparison to DG2 (supplemental figure 5D) to *de novo* GM1 (i.e. CTB-blocked, neuraminidase-treated tissue; supplemental figure 5D), suggests that not all the *de novo* GM1 is available for DG1 binding. One explanation for this would be that *de novo* GM1 might itself form inhibitory *cis* interactions with remaining *a-series* gangliosides (e.g. GM2, GM3) in the plasma membrane of GD3s-KO mice, although this was not addressed experimentally beyond the solid-phase PVDF membrane data shown in Figure 3.19.

GM1 and GD1a co-localize to form an inhibitory environment for DG1 antibody binding in living PC12 cells and in lipid rafts

To investigate the molecular environment that might account for the masking effects of GD1a we used rat neuroendocrine PC12 cells which, in their undifferentiated state, express gangliosides including GM1 and GD1a (Townson et al., 2008). DG1 was entirely unable to bind GM1 in living PC12 cells, in contrast to strong binding with CTB, DG2 and the anti-GD1a mAb MOG35 (Figure 3.21), as predicted from the live nerve terminal findings. Neuraminidase treatment resulted in intense DG1 binding to PC12 cells with complete concomitant loss of MOG35 binding (Figure 3.21B) under conditions in which cell viability was maintained, as monitored by ethidium homodimer-1 staining (data not shown). In co-localization studies of GM1 and GD1a, performed by comparing the binding of the mAbs DG2 and MOG35, the pixel by pixel correlation coefficient was 0.96, indicating very close proximity at the light microscopy level (Figure 3.21C). These findings in PC12 cells indicate a similar PC12 membrane interaction between GM1 and GD1a similar to that occurring in the mouse motor nerve terminal membrane and thereby suggest that this *cis*-inhibitory behaviour may be common other plasma membranes.

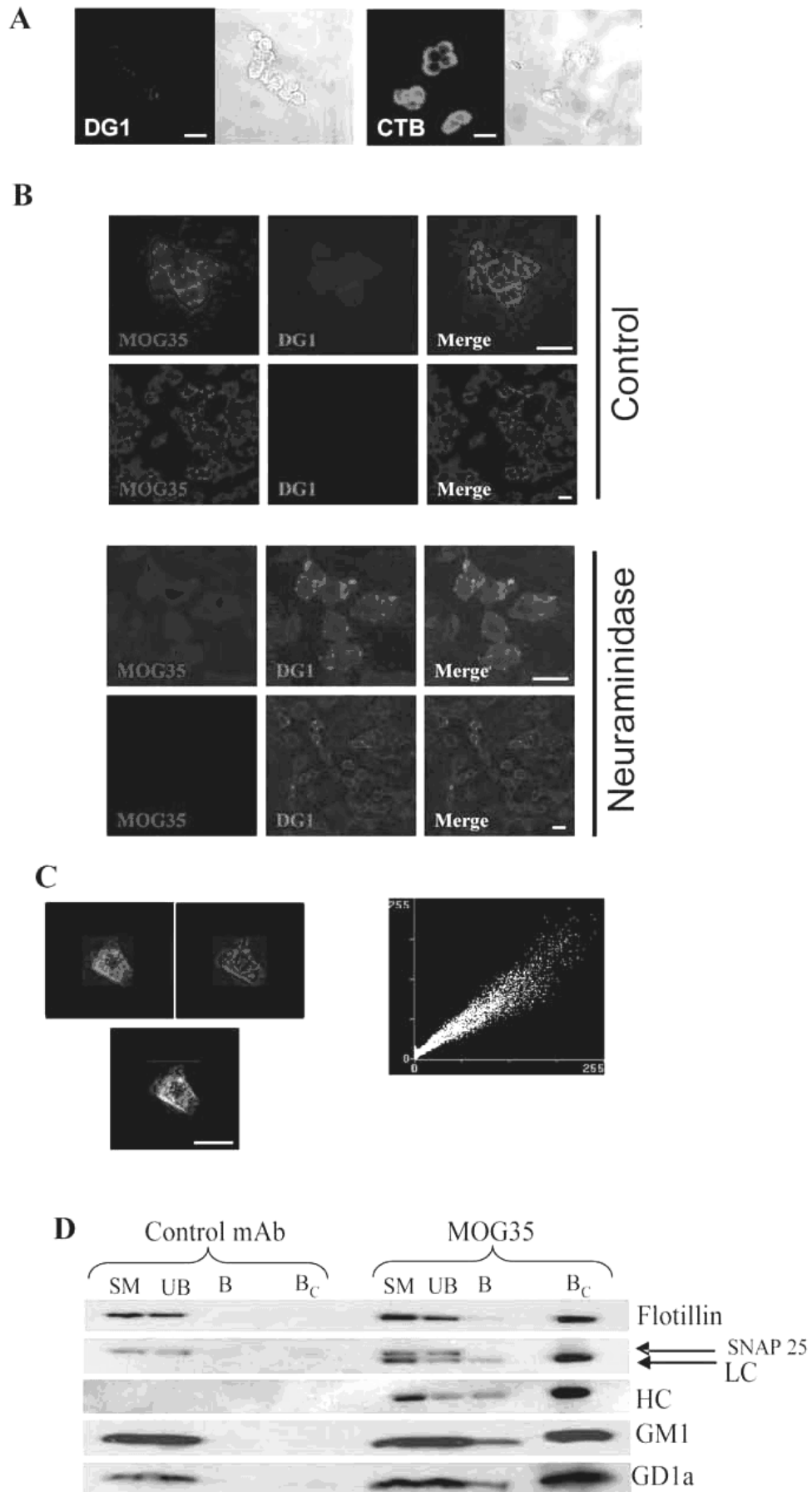
Since gangliosides are enriched in lipid rafts (Simons and Ikonen, 1997), we considered that the raft microenvironment might favour interactions between GM1

and GD1a that were inhibitory for DG1 binding. First we disrupted rafts in live PC12 cells with the cholesterol-sequestering agent methyl- β -cyclodextrin to establish whether GM1 became available for DG1 binding. This was not observed; indeed a general attenuation of ligand binding was seen (supplemental figure 6A). We next considered that raft-localized (GPI-anchored) proteins might exert a masking effect on GM1 for DG1 binding and investigated this by cleaving them with phosphatidylinositol-specific PLC (PI-PLC). PI-PLC treatment enhanced DG2 and MOG35 binding, suggesting that GPI anchored proteins can exert a general and non-specific masking effect on both GM1 and GD1a, but had no significant effect on DG1 binding which remained negligible or absent (supplemental figure 6B). We also conducted the PI-PLC study at live NMJs and observed the same trend as seen in PC12 cells (supplemental figure 6C).

In order to support a molecular interaction hypothesis between GM1 and GD1a, it would be necessary to show that they co-localize in the same raft environment. To investigate this, we incubated PC12 cells with the anti-GD1a mAb MOG35 and an irrelevant isotype control, EG1 (which reacts with GQ1b, GT1a and GD3 but does not bind to PC12 cells; data not shown) and examined GD1a affinity-purified raft fractions by Western blot for GM1 content (Figure 3.21D). This demonstrated that at least a proportion of GM1 was present in the GD1a-enriched raft compartment. In control studies, EG1-affinity purification of raft fractions did not isolate any raft material (as assessed by raft protein markers) or yield any GM1 or GD1a signal. The raft fraction containing GD1a and GM1 also contained the raft marker flotillin, whereas SNAP25 was undetectable, indicating that a heterogeneous population of rafts exist with respect to protein and ganglioside composition in PC12 cells. This latter point was not investigated further.

Figure 3.21. Localization of GM1 and GD1a to raft fractions in PC12 cells (next page).

A. PC12 cell immunostaining (original magnification, x40). DG1 binding to PC12 cells (shown in phase contrast) is not detectable despite the presence of GM1, as shown by CTB staining of cells. Scale bars: 15 μ m. **B.** Effect of neuraminidase on DG1 and MOG35 binding to PC12 cells. Double staining reveals that control cells are positively stained with MOG35 (TRITC), with no binding of DG1 (FITC). Following neuraminidase (N^dase) treatment, MOG35 staining is diminished alongside a concomitant increase in DG1 binding. Scale bars: 15 μ m (images acquired at x40 magnification). **C.** GM1 and GD1a pixel-by-pixel co-localization. FITC and TRITC (anti-GM1 and anti-GD1a respectively) images (x 63 magnification) from double-stained PC12 cells, with co-localization appearing as yellow overlap. Scale bar represents 15 μ m. Plane by plane co-localization (linear scatter plot) shows strong co-localization. **D.** Western blot of raft immunoprecipitation based on MOG35 binding, allowing isolation of GD1a-positive rafts by anti-mouse IgG-coated beads. In irrelevant antibody-incubated cells, no rafts were isolated by anti-mouse IgG-coated beads. Bound sample was concentrated (x10) to amplify any potentially weak signal. In MOG35-incubated cells, a population of rafts was isolated by the beads. Isolated fractions contained the raft-associated protein flotillin, but not SNAP25, which was taken as evidence that the raft extraction procedure did not lead to coalescence of the heterogeneous raft population. Bound fractions also contained both the light chain (LC) and heavy chain (HC) of the anti-GD1a antibody, and the isolated rafts were positive for both GM1 and GD1a. SM, starting material, UB, unbound, B, bound, Bc, bound concentrated.



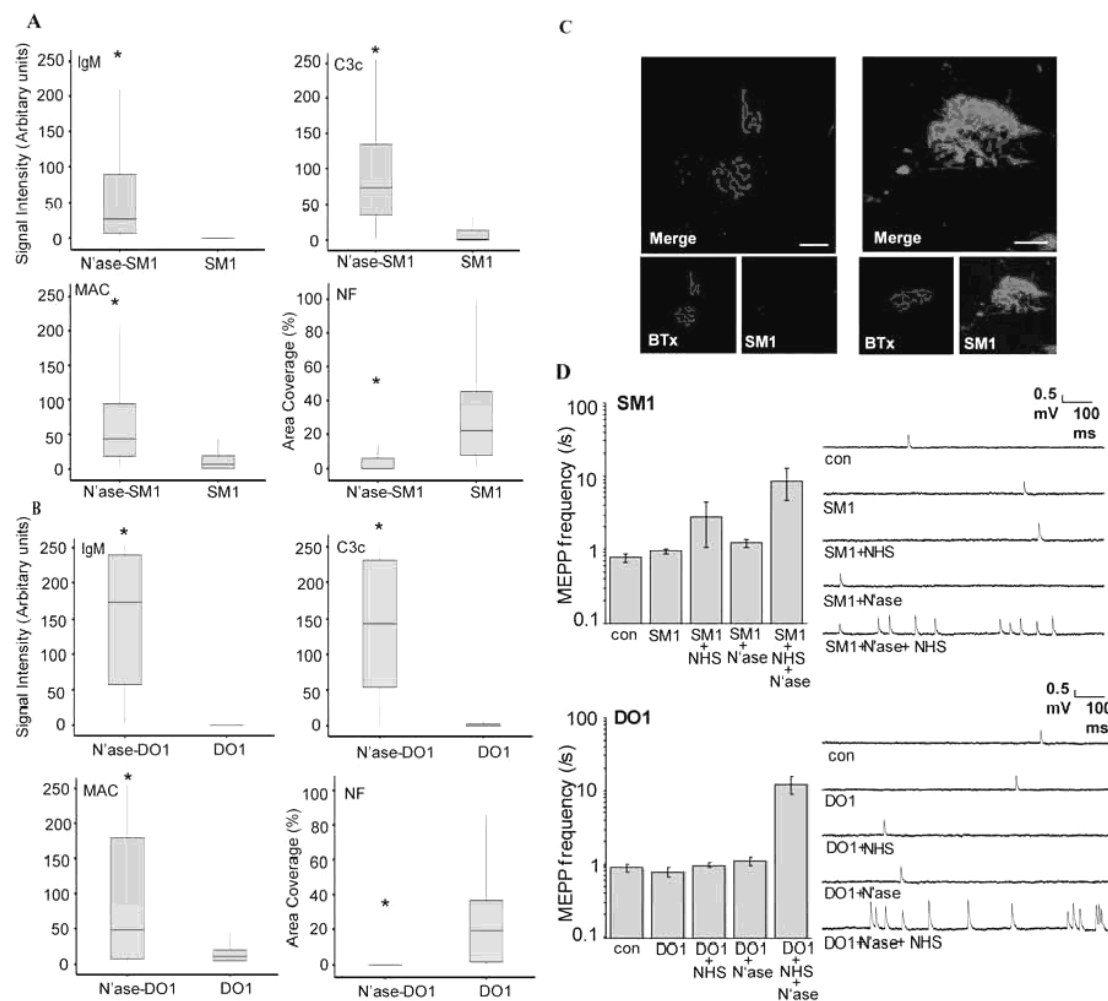


Figure 3.22. Binding and neuropathophysiological effects of human anti-GM1 mAbs SM1 and DO1

A. *Ex vivo* hemi-diaphragm preparations from GD3s-KO mice, as described for Figure 3.18. SM1 only binds NMJs following neuraminidase treatment and then causes a complement-mediated neurofilament (NF) loss. **B.** The experiment was performed as described for **A.**, but with DO1. **C.** Absence of SM1 staining at GD3s-KO triangularis sterni NMJs (left), and presence of staining when the tissue was pre-incubated in neuraminidase (N'dase; right). **D.** Electrophysiological analysis of the effects of SM1 and DO1 at GD3s-KO hemi-diaphragm NMJs, as described in Figure 3.20 for mouse mAb DG1. Following neuraminidase treatment, both SM1 and DO1 bind at NMJs and cause a complement-dependent increase in MEPP frequency.

Scale bars: 20 μ m. Error bars represent SEM for 3 experiments performed in triplicate.

Human neuropathy-associated anti-GM1 mAbs also exert complement-mediated neuropathophysiological effects at the mouse motor nerve terminal, provided GM1 is unmasked

In order to bring clinical relevance to the findings observed with mouse mAbs, we screened human anti-GM1 antibodies cloned from motor neuropathy cases for similar behaviour. Human anti-GM1 IgM antibodies SM1 and DO1 (Paterson et al., 1995) were examined for their binding and complement-mediated neuropathophysiological effects at the mouse NMJ and we assessed a possible inhibitory effect of interaction between GM1 and GD1a (Figure 3.22). Neither mAb bound to GD3s-KO motor nerve terminals, and consequently they did not induce complement deposition (C3c and MAC) or neurofilament loss, as assessed with immunohistochemistry, and did not cause increased MEPP frequency, the electrophysiological hallmark of

neuropathogenicity. However, following neuraminidase pre-treatment of the nerve-muscle preparations, deposits of IgM were massively present. Consequently, the complement cascade was activated, as shown by C3 and MAC deposits, with resultant neurofilament loss and electrophysiological injury as manifested by an increased MEPP frequency.

Discussion

This study has two principle findings. First, we demonstrate that under specific conditions, both human and mouse anti-GM1 antibodies have potent complement activation-mediated neuropathogenic effects at mouse terminal motor axons, including electrophysiological and morphological injury, leading to block of synaptic transmission at the NMJ, thereby resulting in paralysis of the nerve-muscle preparation. Second, we discovered that GM1 ganglioside in living neuronal membranes is masked from binding certain classes of anti-GM1 autoantibody by an interaction with GD1a (and presumably other gangliosides) that is able to shield a critical domain of the oligosaccharide head group involved in antibody binding. Consequently, this masking prevented the neuropathophysiological effects of these anti-GM1 antibodies from occurring. These findings have major implications for our understanding of the pathogenic potential of anti-ganglioside antibodies in neuropathy, their use and relevance as autoimmune disease diagnostics, and as ganglioside detection reagents. The epitope masking effect of this interaction is not a feature concerning all anti-GM1 antibodies, but is dependent upon subtle features of the antibody-antigen interaction that confer these characteristics, and needs to be assessed for each antibody on a case by case basis. Importantly, our results demonstrate that the ability of mouse and human anti-GM1 antibodies to bind isolated GM1 in solid phase assays or in frozen tissue sections cannot be used to predict their pathogenic behaviour *in vivo*. Our findings are likely to account for the markedly discrepant effects of anti-GM1 antibodies observed in many previously published models.

Anti-GM1 antibodies are mainly associated with the acute motor axonal variants of GBS and chronic multifocal motor neuropathies (Willison and Yuki, 2002) and our immunohistochemical and electrophysiological findings at motor nerve terminals may provide a neuropathophysiological explanation for a component of the paralytic symptoms in such patients. In support of this as a clinically relevant site, motor nerve terminal degeneration has been shown at NMJs in a motor point muscle biopsy of an acute motor axonal neuropathy variant GBS patient with anti-GM1 antibodies, while sural nerve biopsy was normal (Ho et al., 1997). The terminal portion of motor axons may be relatively vulnerable for autoimmune attack because it is not protected by a blood-nerve barrier, in contrast to more proximal parts of the axon. GM1 is also present at nodes of Ranvier throughout the length of the nerve. Our results certainly do not exclude anti-GM1 binding and complement-mediated neuropathogenic effects at the more proximal parts of the axon, as has been observed in a rabbit neuropathy model (Susuki et al., 2003).

The complement-mediated pathogenic effects of anti-GM1 mAbs at the NMJ shown here are identical to those observed by us in earlier (*ex vivo* and *in vivo*) mouse studies on mAbs against GQ1b ganglioside and on anti-GQ1b positive patient sera (Plomp et

al., 1999; Halstead et al., 2004; O'Hanlon et al., 2001). Furthermore, anti-GD1a antibodies produce these effects, provided the density of GD1a is high, as in neuronal membranes of GD3s-KO mice (Goodfellow et al., 2005). Thus, this neuropathophysiological effect at murine NMJs is a common feature of certain classes of anti-GM1, -GD1b, -GQ1b and -GD1a antibodies. The heterogeneous distribution of clinical effects observed in patients with these anti-ganglioside antibody specificities does not correlate with the common paralytic effect of these antibodies at mouse NMJs. It is thus likely that regional differences in ganglioside expression, co-expression and density in different neural and glial membranes from different anatomical regions in different species are critical determining factors.

The differences in binding and pathogenic properties of individual anti-glycolipid antibodies may in part be attributable to differences in their affinity or avidity, in addition to their precise specificity. If the affinity of an antibody is high enough to compete effectively for a critical oligosaccharide domain that may otherwise be masked by complex formation with a *cis*-interacting glycolipid, then the pathophysiological consequence of any complex formation might be annulled. Whether this range of affinities can be achieved for anti-glycolipid antibodies is unknown. However, in the case of the widely used high-affinity GM1 ligand CTB, the affinity for GM1 is reported as being in the 10^{-10} to 10^{-12} M range (Kuziemko et al., 1996), which is 10^4 - to 10^6 -fold higher than the mAb DG1, used in the current study. Structural studies have demonstrated that CTB binds GM1 through a 2-fingered pinch interaction involving the nonreducing terminal galactose epitope of GM1 and the internal sialic acid, the latter being masked in our complex formation model (Kuziemko et al., 1996). DG1 also requires the presence of this internal sialic acid for binding to GM1. The implication is therefore that the very high affinity of CTB for GM1 is sufficient to disrupt an inhibitory complex that disguises the sialic acid from its binding domain on the toxin. In contrast, the affinity of DG1 for GM1 is substantially lower than CTB which most likely accounts for DG1's inability to compete effectively for the cryptic sialic acid. Thus affinity may be a relevant factor in disrupting glycolipid-glycolipid complex interactions to allow ligand binding in certain situations. The affinities of DG2 and DG1 for GM1 and GA1 were within fivefold of each other and the half maximal binding values for GM1 were very similar, yet they were completely polarized in terms of their pathogenic potential. It is therefore improbable that a simple explanation of affinity or avidity differences, regardless of dependence upon any complex formation, is the critical factor influencing binding in the current setting, but more likely that the pathogenic differences are due to differences in the fine specificity of the 2 antibodies, specifically their dependence upon the internal sialic acid for binding. Other experimental evidence discussed below also strongly supports this view.

Our study underscores the complexities surrounding the topographical organization of glycolipids in living membranes and how these relate to topography in other types of detection systems such as are used in laboratory immunoassays. Considerable effort is currently focused on achieving optimal platforms for assessing protein-glycan interactions and the issues surrounding complex formation need to be considered in this context (Paulson et al., 2006). The cryptic behaviour of glycolipid headgroups in the plasma membrane, as assessed by accessibility to antibodies is a long recognized phenomenon (Hakomori et al., 1968; Lloyd et al., 1992), but its structural basis has never been demonstrated experimentally. This behaviour may in part be dependent upon ganglioside organisation in lipid rafts. In this study, raft disruption with a cholesterol sequestering agent did not allow the DG1 antibody to bind GM1,

indicating that the interaction of GM1 and GD1a is not cholesterol-dependent, as we also observed by solid phase immunoassay. Rather, it appears to be an intrinsic property of the interacting ganglioside species, possibly localized in a glycosphingolipid signalling sub-domain (Iwabuchi et al., 1998). The ganglioside clustering property may be due to the glycosphingolipid hydroxyl groups acting as hydrogen bond donors, allowing side-by-side interactions within a microdomain (Hakomori, 2002). However, gangliosides require substantial surface area to host their oligosaccharide chain and its associated hydration water, inferring that complex headgroups such as GM1 and GD1a will have large space requirements in the membrane (Sonnino et al., 2007), the consequence of which is unknown in terms of influencing their *cis*-interaction.

Our observations in the GD3s-KO mouse implicate GD1a as the ganglioside that masks GM1 because in this artificial context these are the two predominant gangliosides. Our data on WT mice and in solid-phase assays also strongly suggest that other gangliosides (but not necessarily all gangliosides) may be capable of mediating this masking effect. In the GD3s-KO mouse, the GM1 concentration is substantially higher than in wild-type mice, thereby adding support to the view that the failure of DG1 to bind sialidase-naïve WT or GD3s-KO tissue cannot be simply ascribed to limited availability of GM1 ligand.

The heterogeneity of ganglioside and protein content in lipid rafts means there will be a wide range of raft-associated molecules which could potentially interact with GM1 or inhibit binding of anti-GM1 antibodies through steric hindrance. Our observation that cleavage of GPI anchored proteins resulted in increased DG2 and CTB binding suggests that these proteins have a general masking effect for GM1, although this was not the particular masking effect that prevented the binding of DG1, because that remained at the same low level.

Anti-GM1 mAbs cloned from neuropathy patients were also masked from binding GM1 and therefore unable to bind nerve terminals in *ex vivo* nerve-muscle preparations in these studies. This indicates that the observations with the mouse monoclonal anti-GM1 antibody DG1 is likely to be clinically relevant in man. Further studies are needed to explore the diversity of anti-GM1 antibodies seen in different patient populations to determine the extent of diversity in the context of ganglioside-interaction mediated inhibition. In recent experiments, we have identified anti-GM1 positive neuropathy patient sera that are capable of inducing the neuropathophysiological effects at NMJs as observed here, but only after neuraminidase treatment (J.J. Plomp et al., unpublished results). This further shows that clinically relevant anti-GM1 antisera exists that only bind to unmasked GM1. Such human antibodies may only become clinically important in conditions where the topological organization of gangliosides is destabilized, as for instance during nerve regeneration and the mechanisms by which they may or may not induce immunopathology in normal nerve are currently unknown. In regenerating nerve fibres (following experimental transaction injury), it has been shown that growth cones have enhanced activity of a membrane sialidase that acts to reduce complex gangliosides into GM1 that may thereby render GM1 available for complex attenuated antibody binding. Targeted delivery of sialidase to experimental nerve lesions in the rat enhances axon outgrowth, and this may in part be a result of the increased level of GM1 in the regenerating axon (Yang et al., 2006). In addition, the sialidase-mediated decrease of GD1a interrupts the neuronal adhesion of myelin-associated glycoprotein, which normally inhibits regeneration (Vyas et al., 2002), causing disinhibited axonal regeneration. Based on our observations with neuraminidase, it is possible that

unmasking of cryptic GM1 could allow previously harmless DG1-like antibodies to bind to GM1 rich growth cones and therefore become pathogenic through their ability to impair regeneration. There is experimental evidence, at least for anti-GD1a antibodies, that anti-ganglioside antibodies may influence neuronal regeneration (Lehmann et al., 2007). The existence of anti-GM1 antibodies like DG1 that specifically bind regenerating neural tissue and cause complement-mediated injury would have significant clinical consequences in that it could be the start of recurrent cycles of axonal injury and regeneration. Therefore, rapid and persistent clearance of such antibodies should be an important therapeutic goal.

It is possible that GM1 masking occurs by interaction with gangliosides other than GD1a. Indeed, in our control experiments the Gal(β 1-3)GalNAc moiety of GD1b was apparently shielded from binding DG2, whereas MOG1 - which presumably binds at least in part the internal disialosyl structure of GD1b as it is GD1b monospecific - was able to bind GD1b. Studies showing neuropathic effects restricted to sensory ataxia due to mono-specific anti-GD1b antibodies in a rabbit model are consistent with this finding (Kusunoki et al., 1999), as are recent studies using human ataxic GBS sera (Kaida et al., 2008a). We did not explore further the nature of this antibody-binding inhibitory interaction of GD1b with other gangliosides.

With regard to the induction of anti-GM1 antibodies by *C. jejuni* infection in GBS, our findings illuminate the protective role of antibody in microbial defence, as balanced against the development of autoimmune injury. Thus, DG1 is able to bind the GM1 mimic in LOS on live *C. jejuni* that bears also a GD1a mimic in a 50:50 ratio, indicating that a masking interaction does not occur. This shows that DG1-like antibodies could be bactericidal without binding to neuronal GM1, and thus (at least initially) would not exert an autoimmune neuropathic effect. Furthermore, DG1-like-producing B-cell clones do not recognize self-GM1 in living membranes. Therefore, breaking tolerance to GM1 might not be required in order to generate protective immunity against self-mimicking microbial molecules.

We were able to reproduce a masking effect of GD1a and other gangliosides on GM1 in immunoassays conducted either on polystyrene plates or PVDF membranes, recapitulating the discordant findings observed in living membranes between DG1 and DG2. Interestingly, the inhibitory complexes between GM1 and GD1a form on both polystyrene and PVDF immobilization platforms and thus the molecular interactions between GM1 and other gangliosides must spontaneously form when they are admixed, in addition to being organized or disorganized in living or dead membranes, respectively. Furthermore, these solid phase immunoassays demonstrated that interaction is not unique to GM1 and GD1a but can also occur between GM1 and other gangliosides. In addition, they form important support for the conclusion drawn from our neuraminidase experiments in live tissues - that the enabling factor for DG1 binding to the neuronal membrane under these conditions is an unmasking of pre-existing GM1 by the removal of the external neuraminic acid of GD1a, as opposed to simply an increase in GM1 density by creation of de novo GM1 from GD1a.

Based on these experimental findings, we conclude that the micro-heterogeneity of ganglioside binding by anti-GM1 antibodies substantially influences their neuropathogenic potential, and that the basis for these differences is likely due to subtle conformational arrangements within the membrane, leading to complexes of GM1 with other gangliosides that inhibit binding of some types of anti-GM1 antibodies. These ganglioside interactions appear to be stably formed in living membranes, but are highly susceptible to disintegration (and thus epitope exposure) in frozen or otherwise altered tissue. The changes in the molecular architecture of the

living and dead/disrupted membrane that underlie these properties are unknown and difficult to investigate at the molecular biophysical level. In solid-phase immunoassays, these gangliosides interactions are also able to form. Recent studies showed that complexes of two different gangliosides can form neo-epitopes that specifically bind certain neuropathy-associated antibodies which do not bind to the single ganglioside species (Willison, 2005; Kaida et al., 2004b; Kaida et al., 2006). Together with the data provided here this leads to the conclusion that ganglioside interaction may either enable or inhibit antibody binding to the neuronal membrane, or be neutral, depending of the type of circulating antibody. To describe these 3 conditions, we propose the terms *complex-enhanced*, *complex-attenuated*, and *complex-independent*. In the light of these findings, we need to critically evaluate any individual antibody-ganglioside interaction detected by the wide range of techniques available in order to be able to interpret the neuropathogenic potency of such an interaction in the living neuronal membrane.

**Supplemental material is available online with this article;
doi:10.1172/JCI37338DS1**

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 glycosphingolipids, 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 neuropathophysiological 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 $\text{Ca}_v2.1$ 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 needs 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 this 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.

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Nederlandse samenvatting

**De rol van gangliosiden en anti-ganglioside
antistoffen in de werking van de
neuromusculaire synaps**

Het syndroom van Guillain-Barré (GBS) is een acute, postinfectieuze neuropathie met een monofasisch verloop. De ziekte kent verscheidene klinische vormen, maar algemeen zijn de symptomen van symmetrische spierzwakte en afwezigheid van reflexen. Een van de mildere klinische varianten van GBS is het Miller Fisher syndroom, welk gekenmerkt wordt door ataxie, areflexie en verlamming of verzwakking van de externe oogspieren. In meer dan de helft van de GBS patiënten kan in het serum de aanwezigheid worden aangetoond van antistoffen tegen gangliosiden. Gangliosiden behoren tot de glycosphingolipiden en bevatten één of meer siaalzuren. Ze vormen een onderdeel van de buitenste laag van de celmembranen en komen voor in elk celtype. Echter, in de membranen van zenuwcellen en dan in het bijzonder in het presynaptische membraan aan het uiteinde van axonale zenuwceluitlopers zijn gangliosiden in verhoogde mate aanwezig. De grote familie van gangliosiden kent een specifieke stapsgewijze synthese, waarbij ceramide de basisstructuur vormt en door toevoeging van suikergroepen en siaalzuren de verschillende familieleden opgebouwd worden. Gangliosiden spelen een rol in cel-cel herkenning en communicatie. Bovendien beïnvloeden ze allerlei processen in het gehele lichaam, waaronder apoptose en spermatogenese. Mede omdat gangliosiden zo geconcentreerd zijn in het presynaptische membraan, wordt verondersteld dat zij betrokken zijn in synaptische transmissie en dan in het bijzonder in het proces van neurotransmitter afgifte. In dit proefschrift is met behulp van onder andere elektrofysiologie de fysiologische rol die deze gangliosiden spelen in neurotransmitter afgifte onderzocht in neuromusculaire synapsen van muizen. Daarnaast zijn in deze synapsen ook de pathofysiologische effecten bestudeerd van antistoffen tegen deze gangliosiden zoals die worden gevonden in serum van GBS patiënten.

In het eerste deel van dit proefschrift is gekeken naar de functie van gangliosiden in het proces van neurotransmitter afgifte in de neuromusculaire synaps van de muis. In hoofdstuk 2.1 zijn synapsen van twee verschillende ganglioside knock-out muizen met behulp van elektrofysiologische technieken onderzocht. Bij deze muizen ontbreekt een deel van de synthases die nodig zijn om de familie van gangliosiden op te bouwen en hierdoor is een subset van die familie afwezig. Bovendien zijn de gangliosiden die wel geproduceerd kunnen worden als gevolg van accumulatie of “opregulatie” in hogere concentratie aanwezig. De geteste muizen misten hetzij de b- en c-series van de gangliosiden, hetzij de b- en c-series *en* alle complexe gangliosiden waardoor ze alleen ganglioside GM3 in hun celmembranen hadden (zie Figuur 1.3). We vonden dat neuromusculaire synapsen van deze ganglioside mutanten alleen subtiele verschillen vertonen in de afgifte van neurotransmitter in vergelijking met wildtype controles, zoals een subklinisch lager *steady-state* niveau bij hoogfrequente zenuwstimulatie (dit is de plateaufase in transmitter afgifte die optreedt na een initiële inzakking). Dit suggereert dat gangliosiden wel degelijk een modulerende invloed hebben, maar dat er geen cruciale, onmisbare rol is die om de aanwezigheid van de complete familie van gangliosiden vraagt. Zelfs wanneer alleen het eenvoudige ganglioside GM3 nog aanwezig was, vertoonde de neurotransmitter afgifte slechts een geringe reductie ten opzichte van het wildtype en was de synaps nog steeds succesvol in het overbrengen van impulsen van zenuw- naar spiercel. Omdat deze experimenten uitgevoerd werden in relatief jonge muizen (jonger dan drie maanden) en gangliosiden mogelijk niet zozeer een rol spelen in de vroege (embryonale en postnatale) levensfase, maar wellicht belangrijker zijn voor onderhoud later in het leven, hebben we in hoofdstuk 2.2 synapsen van transgene muizen ouder dan negen maanden nader bekeken. We hebben onderzocht hoe de neuromusculaire synaps van ganglioside

knock-out muizen functioneert op oudere leeftijd, wanneer herstel, onderhoud en regeneratie van belang zijn. Ook bij deze oude muizen was de afwezigheid van subsets van de familie (alle complexe gangliosiden of de b- en c-series gangliosiden) geen belemmering voor het functioneren van de synaps: opnieuw waren er alleen subtiele veranderingen meetbaar die geen gevolgen hadden voor de impulsoverdracht. Dit impliceert dat ofwel gangliosiden geen cruciale rol hebben in het proces van transmitter afgifte, ofwel dat de verschillende gangliosiden elkaars functie kunnen compenseren en dat het overblijven van bijvoorbeeld alleen ganglioside GM3 daarom voldoende is om de synaps werkend te houden. Om dit verder te onderzoeken hebben we, zoals beschreven in hoofdstuk 2.3, dit laatste ganglioside in een *in vitro* experiment “verwijderd” uit het weefsel van een transgene muis die alleen ganglioside GM3 tot expressie kon brengen. Met behulp van het enzym neuraminidase werd van dit laatste ganglioside het siaalzuur afgeknipt, waarmee het onderscheidende kenmerk van de ganglioside-familie verdwenen was. Hierbij vonden we wel enkele grotere veranderingen in de afgifte van neurotransmitter, zoals een *steady-state* niveau bij hoogfrequente zenuwstimulatie dat 20% lager lag dan in een wildtype synaps, waardoor de “compensatie theorie” enigszins lijkt te worden ondersteund. Maar nog steeds was dit effect onvoldoende om de impulstransmissie in de neuromusculaire synaps te blokkeren. Dit onderstreept nog eens dat gangliosiden geen essentiële en fundamentele rol spelen in de afgifte van neurotransmitter, zelfs niet op oudere leeftijd, maar dat ze wel enige modulerende invloed hebben.

Het tweede deel van dit proefschrift focust op de neuropathofysiologische effecten van antistoffen tegen gangliosiden en de therapeutische werking van complementremmers hierop. Hoofdstuk 3.1 beschrijft het veronderstelde werkingsmechanisme van de antistoffen die na binding aan de gangliosiden in het celmembraan in staat zijn om complement te activeren. Deze reactie leidt tot beschadiging van het presynaptische motor-zenuwuiteinde en heeft tot gevolg dat Ca^{2+} -ionen vrij het uiteinde van de zenuw in kunnen diffunderen. Omdat Ca^{2+} -ionen de afgifte van neurotransmitter stimuleren, is het resultaat een ongecontroleerde, massale afgifte van neurotransmitter wat uiteindelijk leidt tot uitputting van de zenuw en verlamming van de spier door blokkade van neuromusculaire transmissie. Omdat dit neuropathofysiologische proces complement afhankelijk is, lijkt het blokkeren van het complementsysteem een doeltreffende manier om in te grijpen en het schadelijke effect tot stilstand te brengen. In de hoofdstukken 3.2 en 3.3 zijn twee remmers van het complement systeem getest op hun effectiviteit om het presynaptische membraan te beschermen tegen de beschadigende effecten van de anti-ganglioside antistoffen. De eerste remmer is een gehumaniseerd muizenmonoklonaal antilichaam, eculizumab, en de tweede is een recombinant eiwit gebaseerd op een eiwit dat is geïsoleerd uit het speeksel van de zachte teek *Ornithodoros moubata*. Beide blokkers remmen de splitsing van complement component C5 in C5a en C5b. C5b is de eerste component van het *membrane attack complex* (MAC), dat samen met C6-C9 wordt gevormd. MAC is een moleculair complex dat gaten maakt in het celmembraan met lysis van de cel tot gevolg. Dit is een cruciale stap in het beschadigende effect dat optreedt als gevolg van de binding van antistoffen aan de gangliosiden. Door de formatie van MAC te voorkomen waren beide complement remmers in staat om de neuropathologische effecten van de antistoffen te remmen in zowel een *in vitro* als een *in vivo* muismodel voor het Miller Fisher syndroom. Deze stoffen zijn daarom interessante kandidaten om in neuropathie patiënten te testen. Naast effecten als gevolg van de activatie van complement door de anti-ganglioside antistoffen hebben

we ook gekeken naar de verschillende typen antistoffen die deze immuunreactie op gang kunnen brengen (hoofdstuk 3.4). Enkele jaren geleden is ontdekt dat er niet alleen antistoffen bestaan die binden aan epitopen op de individuele gangliosiden, maar ook antistoffen die binden aan combinaties van twee verschillende gangliosiden, die kennelijk samen één, geheel nieuwe bindingsplaats vormen voor antistoffen (een ganglioside complex). In een oriënterende studie hebben wij een aantal serummonsters met anti-complex antilichamen getest op hun potentie om neuropathofysiologische effecten te veroorzaken in de neuromusculaire synaps van de muis, zoals eerder aangetoond met sera met antistoffen tegen individuele gangliosiden. Ongeveer de helft van de 27 onderzochte monsters had een beschadigend effect, blijkend uit elektrofysiologische experimenten. Echter, de mate waarin de monsters effecten sorteerden was zeer gevarieerd en bovendien werd de interpretatie bemoeilijkt door het feit dat veel sera ook in meer of mindere mate additionele antistoffen bevatten tegen individuele gangliosiden. De ontwikkeling van monoclonale antistoffen met monospecificiteit tegen ganglioside complexen zal verder onderzoek naar de exacte rol van anti-ganglioside complex antistoffen faciliteren.

Tenslotte is in hoofdstuk 3.5 nader gekeken naar de condities waaronder anti-ganglioside GM1 antistoffen in staat zijn om aan ganglioside GM1 te binden. Het is al eerder gebleken dat het niet alleen belangrijk is dat de gangliosiden aanwezig zijn in het membraan, maar ook in welke confirmatie ze aanwezig zijn en welke interacties ze aangegaan zijn met naburige moleculen. Deze studie laat zien dat een ganglioside in staat is om de bindingsplaats voor antistoffen bij een naburig GM1 ganglioside te maskeren. Het is afhankelijk van de structuur van de antistof of deze vervolgens nog kan binden of niet. Als gevolg van deze interactie tussen naburige gangliosiden kan de activiteit van verschillende antistoffen gericht tegen eenzelfde ganglioside drastisch variëren. Tevens werden in deze studie voor het eerst neuropathofysiologische effecten van (patiënten- en muizen-) anti-GM1 antistoffen op de neuromusculaire synaps aangetoond.

Concluderend laten de studies in dit proefschrift zien dat gangliosiden niet essentieel lijken te zijn voor het functioneren van de neuromusculaire synaps met betrekking tot transmitter afgifte, maar wel een modulerende rol hebben. Daarnaast blijkt het blokkeren van het complement systeem met behulp van C5-remmers een effectieve methode om zenuwmembranen te beschermen tegen de beschadigende effecten van complement activatie na binding van anti-ganglioside antistoffen. In het laatste hoofdstuk worden deze en andere experimentele bevindingen in dit proefschrift bediscussieerd in het licht van de bestaande literatuur en de pathogenese en therapeutische aspecten van GBS.

List of Abbreviations

α BTx	α -bungarotoxin
α LTx	α -latrotoxin
ACh	acetylcholine
AChR	acetylcholine receptor
AIDP	acute inflammatory demyelinating polyradiculoneuropathy
AMAN	acute motor axonal neuropathy
AMSAN	acute motor-sensory neuropathy
BBE	Bickerstaff's brainstem encephalitis
BSA	bovine serum albumin
C	complement
Ca _v 1	voltage-gated calcium channel type 1 (L-type calcium channel)
Ca _v 2.1	voltage-gated calcium channel type 2.1 (P/Q-type calcium channel)
CFP	cyan fluorescent protein
<i>C. jejuni</i>	<i>Campylobacter jejuni</i>
CIDP	chronic inflammatory demyelinating polyradiculoneuropathy
CMV	cytomegalovirus
CNS	central nervous system
Crry	complement receptor-1 related gene/protein Y
CTB	cholera toxin B-subunit
DAF1	decay accelerating factor 1 (also: CD55)
(d)KO	(double) knockout
EAN	experimental autoimmune neuritis
EC50	half maximal effective concentration
EM	electron microscopy
EPP	endplate potential
EthD-1 / EthD-2	ethidium homodimer-1 / 2
fH	Factor H
GalNAc(-T)	β 1,4-N-acetylgalactosaminyl(transferase)
GBS	Guillain-Barré syndrome
GD3synthase	α 2,8sialyltransferase
GFP	green fluorescent protein
GM2synthase	see: GalNAc-T (also: GM2/GD2synthase)
IgG / IgM	immunoglobulin type G / type M
IVIg	intravenous immunoglobulin
KDO	3-deoxy-D-manno-2-octulosonic acid
LacCer	lactosylceramide
LOS	lipooligosaccharide
LPS	lipopolysaccharide
(m)Ab	(monoclonal) antibody
MAC	membrane attack complex; complement components C5b to C9
MAG	myelin-associated glycoprotein
MASP	MBL-associated serine protease
MBL	mannose-binding lectin
MEPP	miniature endplate potential
MFS	Miller Fisher syndrome
nAChR	nicotinic acetylcholine receptor
NeuAc	N-acetylneuraminic acid
NeuGc	N-glycoloyl-neuraminic acid
NF	neurofilament
NHS	normal human serum
NMDA	N-methyl-D-asparaginezuur
NMJ	neuromuscular junction
PBS	phosphate-buffered saline
PNS	peripheral nervous system
pSC	perisynaptic Schwann cell
PVDF	polyvinylidene difluoride
RRP	readily releasable pool
SNAP25	synaptosomal-associated protein of 25kDa
SNARE	soluble N-ethylmaleimide-sensitive factor attachment protein receptor
VGCC	voltage-gated Ca ²⁺ channel
WT	wildtype
QC	quantal content

List of Publications

Greenshields K, Halstead SK, Zitman FMP, Rinaldi S, Brennan KM, O’Leary C, Chamberlain LH, Easton A, Roxburgh J, Pediani J, Furukawa K, Furukawa K, Goodyear CS, Plomp JJ, and Willison HJ

The neuropathic potential of anti-GM1 autoantibodies is regulated by the local glycolipid environment

Journal of clinical investigation, 2009;119(3):595-610.

Halstead SK*, Zitman FMP*, Humphreys PD, Greenshields K, Verschuuren JJ, Jacobs BC, Rother RP, Plomp JJ, and Willison HJ

Eculizumab prevents anti-ganglioside antibody-mediated neuropathy in a murine model

Brain, 2008;131(Pt 5):1197-208

* These authors contributed equally to this work

Halstead SK, Humphreys PD, Zitman FMP, Hamer J, Plomp JJ, and Willison HJ

C5 inhibitor rEV576 protects against neural injury in an in vitro mouse model of Miller Fisher syndrome

Journal of the peripheral nervous system, 2008; 13(3):228-35

Willison HJ, Halstead SK, Beveridge E, Zitman FMP, Greenshields KN, Morgan BP, and Plomp JJ

The role of complement and complement regulators in mediating motor nerve terminal injury in murine models of Guillain-Barré syndrome

Journal of Neuroimmunology, 2008; 201-202:172-82

Zitman FMP, Todorov B, Jacobs BC, Verschuuren JJ, Furukawa K, Furukawa K, Willison HJ, and Plomp JJ

Neuromuscular synaptic function in mice lacking major subsets of gangliosides

Neuroscience, 2008; 156(4):885-97

Zitman FMP, Todorov B, Verschuuren JJ, Jacobs BC, Furukawa K, Furukawa K, Willison HJ, and Plomp JJ

Neuromuscular synaptic transmission in aged ganglioside-deficient mice

Neurobiology of Ageing, 2009; (doi:10.1016/j.neurobiolaging.2009.01.007)

Zitman FMP, Todorov B, Furukawa K, Furukawa K, Willison HJ, and Plomp JJ

Total ganglioside ablation at mouse motor nerve terminals alters neurotransmitter release level

Synapse 2010; 64:335-338

Curriculum Vitae

Femke Zitman werd geboren op 24 april 1979 te Leiden. In 1997 behaalde zij haar gymnasium diploma aan het Stedelijk Gymnasium te Nijmegen en is zij begonnen met de studie Industrieel ontwerpen aan de TU Delft. Gaandeweg ontdekte zij dat haar interesse toch meer lag bij de biologie en de biomedische wetenschappen en na het behalen van de propedeuse is zij daarom in 1999 overgestapt naar de studie Biologie. Het eerste jaar Biologie heeft zij gevolgd aan de UvA te Amsterdam en daarna heeft zij de studie afgemaakt aan de Universiteit van Leiden. Als onderdeel van de opleiding heeft zij een onderzoeksstage uitgevoerd bij TNO *Voeding en gezondheid* te Zeist gedurende driekwart jaar en daarna volgde nog een stage bij de afdelingen Bioinformatica en Evolutiebiologie aan de Universiteit van Leiden. Zij heeft zich gespecialiseerd in de Medische Biologie en in de Bioinformatica en in 2004 heeft zij voor beide richtingen haar doctoraal diploma behaald. In 2005 is zij begonnen aan haar promotieonderzoek op de afdelingen Neurologie en Moleculaire Celbiologie van het LUMC te Leiden onder leiding van Dr. J.J. Plomp. De resultaten van dat onderzoek staan beschreven in dit proefschrift. In 2009 heeft zij het onderzoek afgerond en is begonnen aan een postdoc project aan de Universiteit van Haifa te Israël bij de onderzoeksgroep van Prof. Dr. G. Richter-Levin.
