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Gangliosides and anti-ganglioside antibodies in neuromuscular synaptic function

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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.

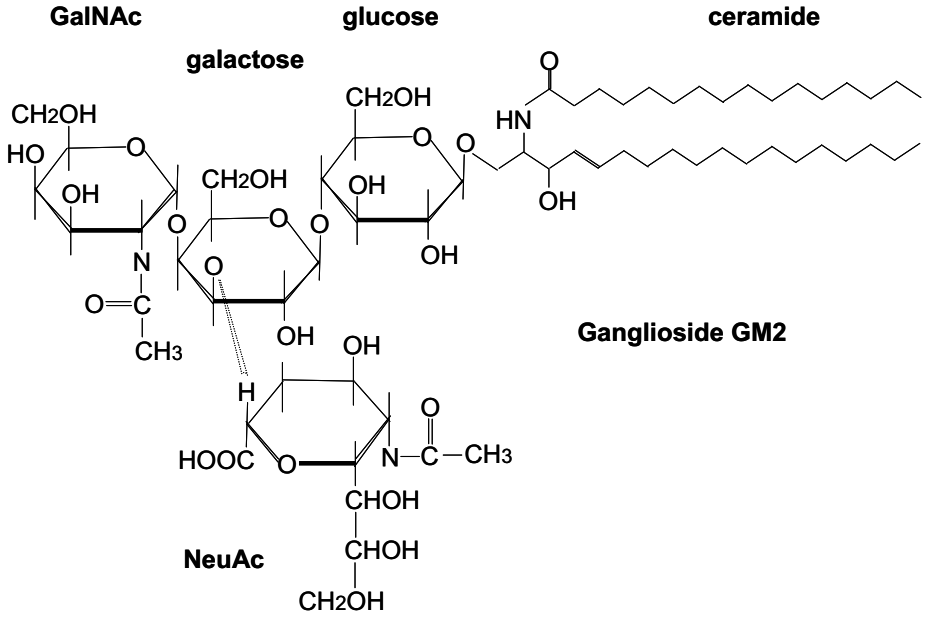

Figure 1.1. Structure of ganglioside GM2

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 endoplasmatic 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).

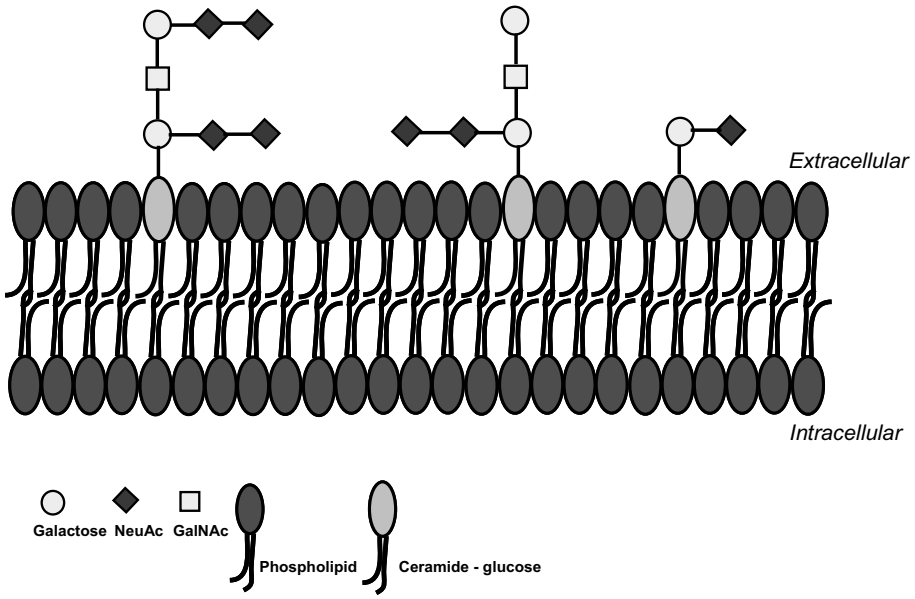

Figure 1.2. Schematic overview of gangliosides inserted in the phospholipid bilayer of the cell membrane. The hydrophilic portions of the gangliosides protrude into the extracellular space.

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.

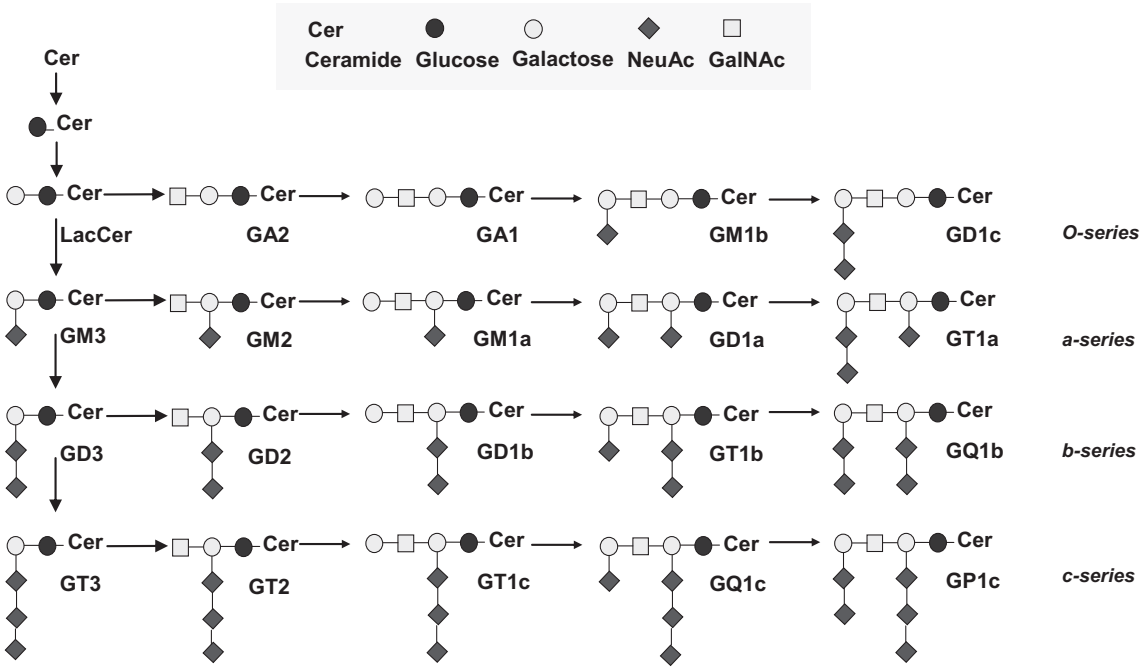

Figure 1.3. Simplified scheme of the ganglioside stepwise biosynthesis. Arrows point in the direction of the sequential step in the synthesis.

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 GQ1bα 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.

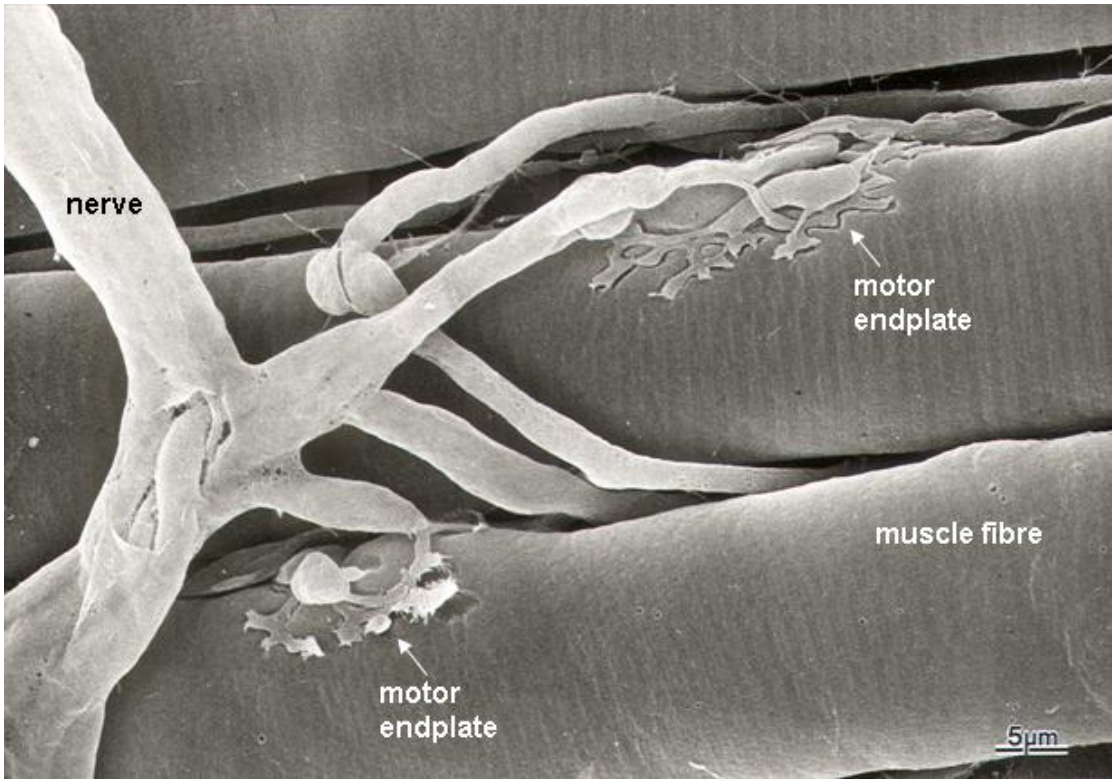

Figure 1.4. Scanning electron micrograph of two NMJs in the sternothyroid muscle of the Chinese hamster
The intramuscular nerve produces side branches, which terminate in motor end-plates on the muscle nerve surface. Picture is used with approval of Dr. Desaki.

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 actives 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 $Ca_v2.1$-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).

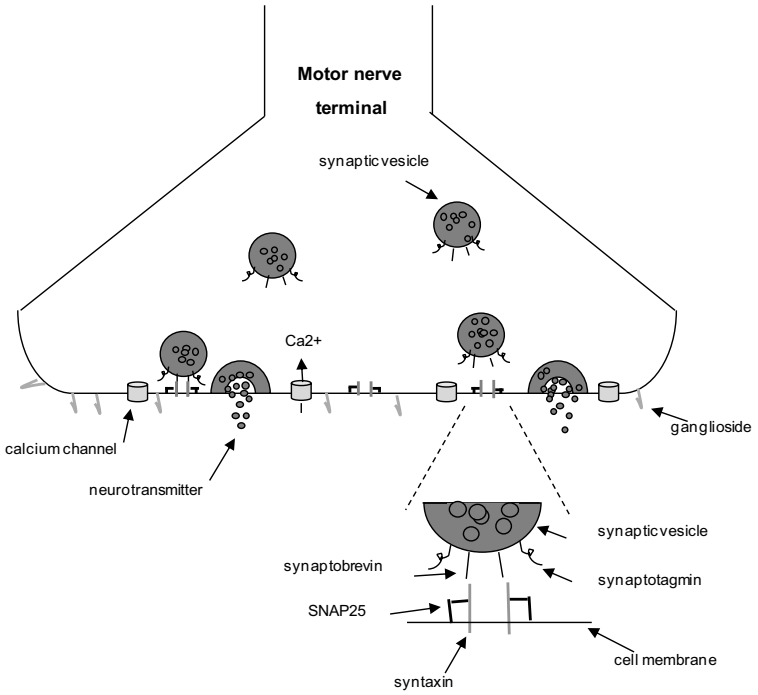

Figure 1.5. Neurotransmitter release at the motor nerve terminal

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*.

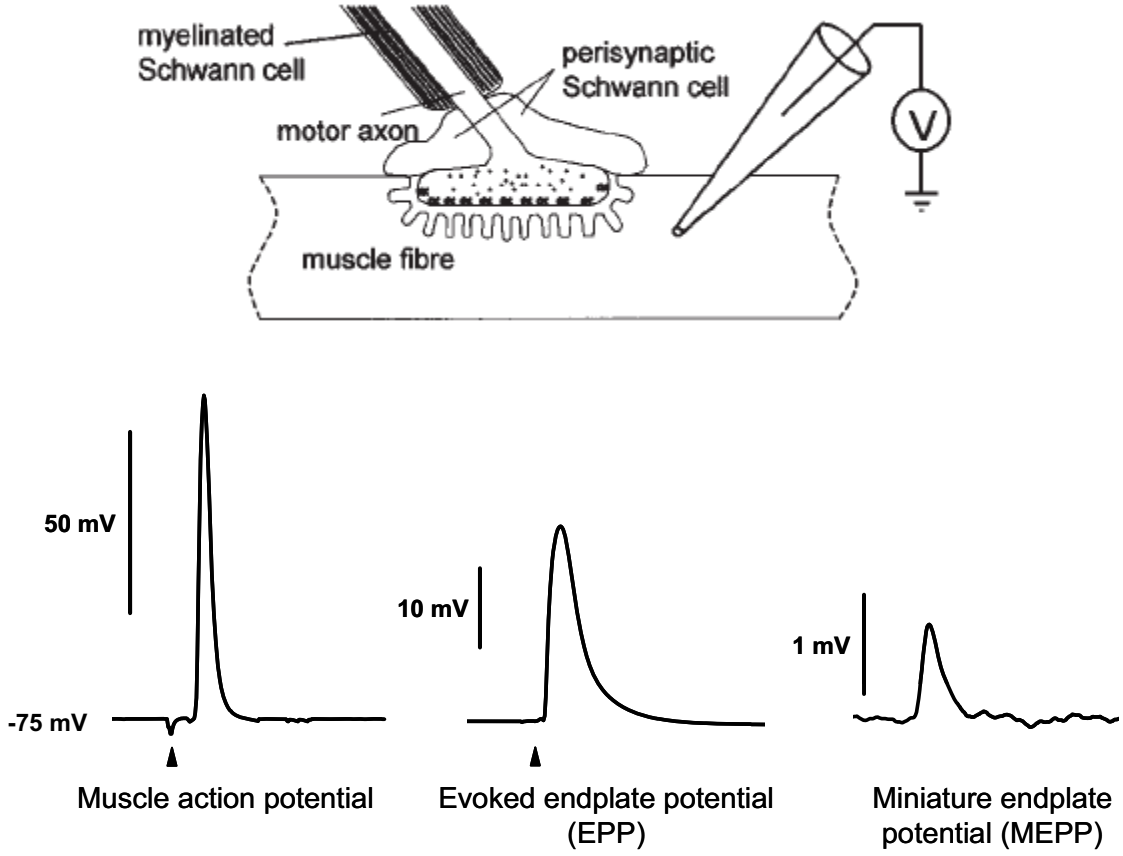

Figure 1.6. Measurement at the NMJ
Top scheme of motor endplate is adapted from O'Hanlon et al. (2002) and shows the micro-electrode (on the right) inserted into the muscle fibre. This way, alterations in the membrane potential of the muscle fibre can be recorded. These alterations are the result of 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 ~1 μm 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 paralyis (Middlebrook and Dorland, 1984; Brunger and Rummel, 2009).
Another neurotoxin that has the NMJ as target site is α-latrotoxin (αLTx). αLTx 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, αLTx 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 αLTx, 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 αLTx 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 αLTx-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, opthalmoplegia, 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; Notturno 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; Rekand 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).

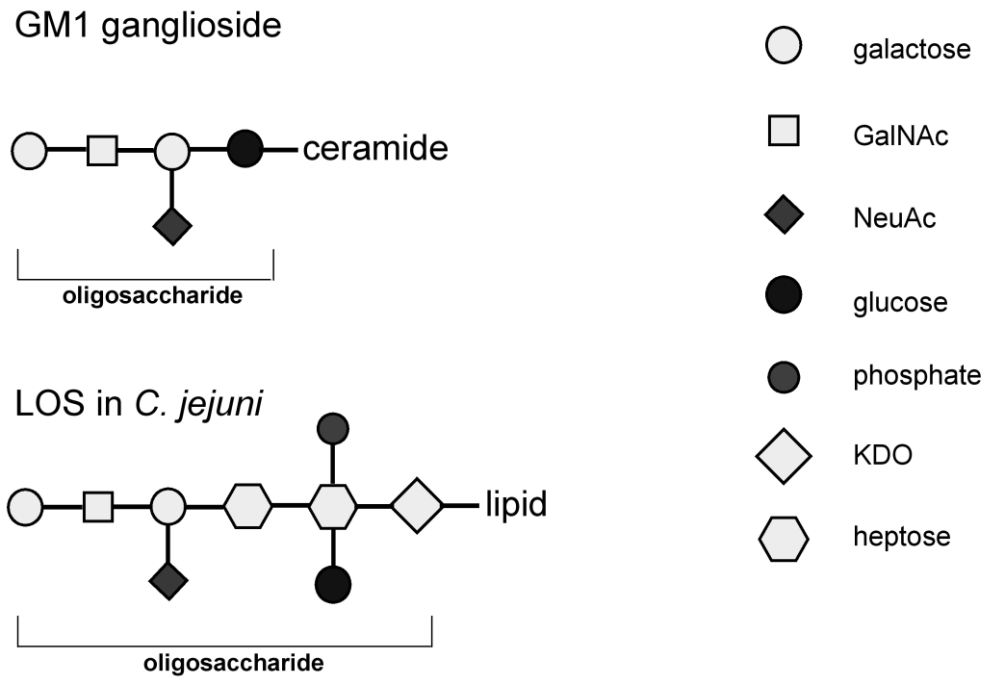

Figure 1.7. Picture of identical structure GM1 and the outer core of *C. jejuni* LOS
KDO: 3-deoxy-D-manno-2-octulosonic acid (picture is based on: Moran and Prendergast, 2001)

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).

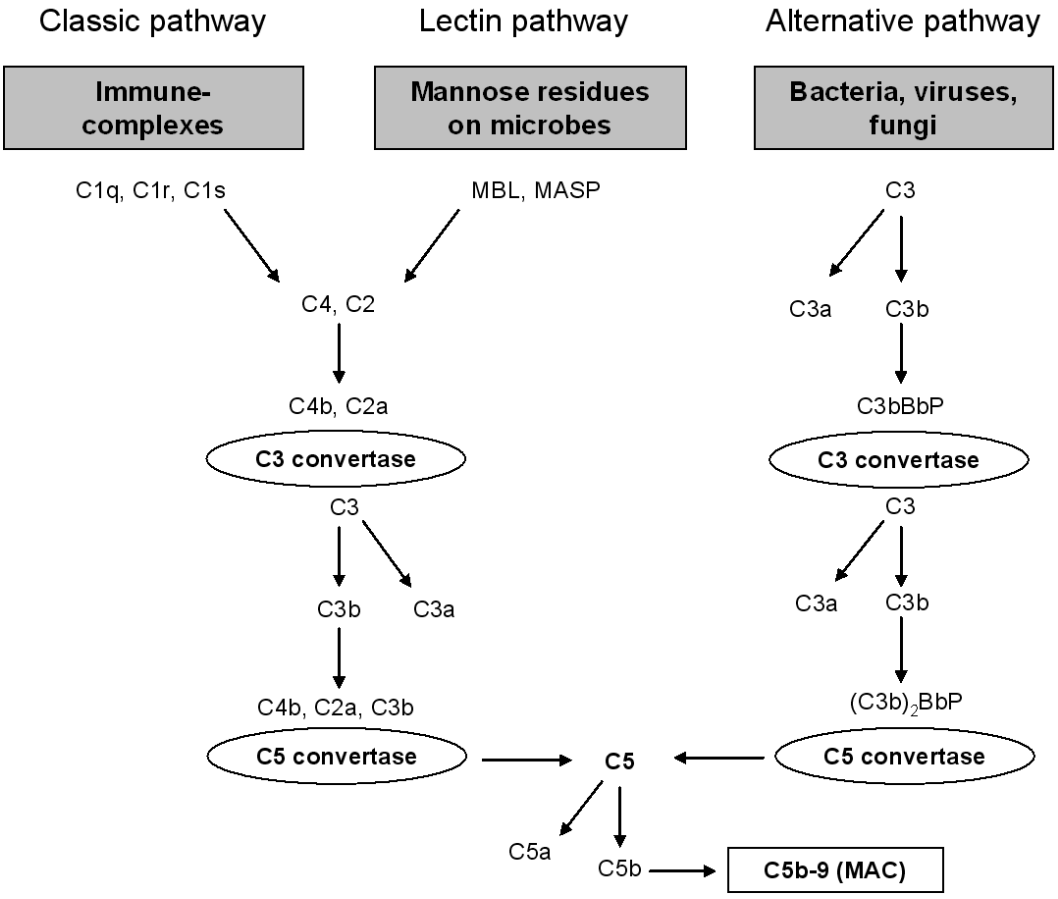

Figure 1.8. Scheme of complement cascade
The classical pathway is mainly activated by antibody-antigen binding. The lectin pathway starts with the binding of *mannose-binding lectin* (MBL) to carbohydrate surfaces and subsequent binding of *MBL-associated serine proteases* (MASPs). The alternative pathway 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 β1,4-*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 α2,8-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 (α2,8sialyltransferase) 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

abnormalities. They do show however a heightened sensitivity to insulin, and therefore need to be on a diet (Yamashita et al., 2003; Sandhoff et al., 2005).
A second combination of gene disruption has lead to the GM2*GM3synthases double knockout mice, which are not able to express the ganglio-series at all. These mice express increased amounts of the biosynthetic precursor, lactosylceramide, and still express the three other glycosphingolipid series, i.e. the lacto-series, the neolacto-series, and the globo-series. Mice are viable, but soon after birth neurodegeneration is observed and the mice develop hind limb weakness, ataxia, and tremors. These abnormalities become more severe with age and the maximal life span is only about 3 months (Yamashita et al., 2005).
Finally, also a mouse has been generated with a disrupted glucosylceramide synthase gene (*Ugcg*), which eliminates the major synthesis pathway of glycosphingolipids. So these mice do not just lack the gangliosides, but also their precursor lactosylceramide and all the other glycosphingolipid series as well. Homozygosity for this gene disruption is embryonically lethal, showing intense apoptosis in the ectodermal layer in gastrulation stage embryos, indicating indispensability of glycosphingolipids for embryonic development (Yamashita et al., 2002). Specific neuronal disruption of the *Ugcg* gene did not impair embryonic development. However, all mice died within 3 weeks after birth from severe neurodegeneration. They had structural defects in the peripheral nerves and in the cerebellum. This indicates sphingolipid requirement for neural maturation and maintenance, and excludes a role for gangliosides in early embryonic development of the nervous system (Jennemann et al., 2005).

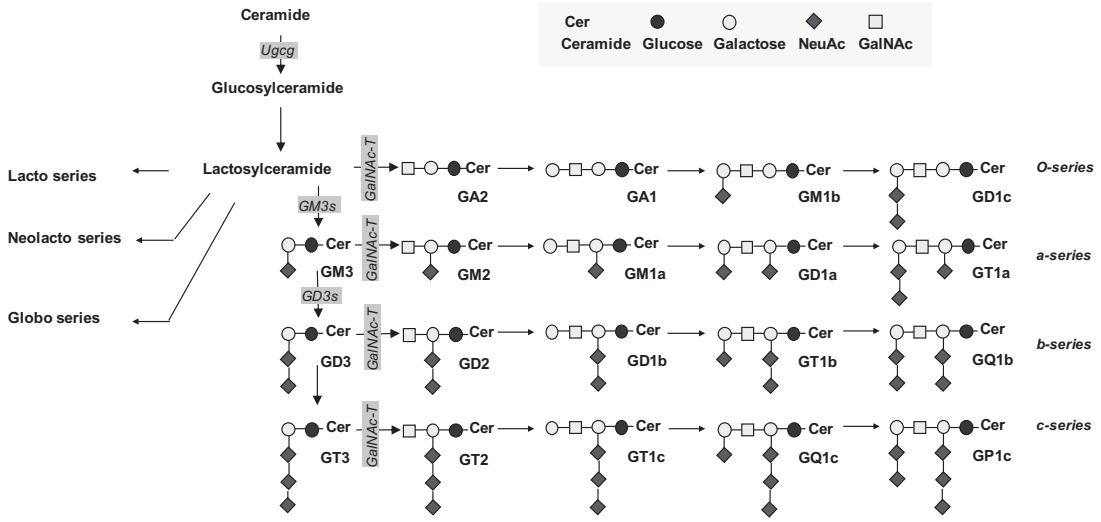

Figure 1.9. Simplified biosynthetic scheme of the gangliosides and their precursors: the names in the grey squares represent key genes that can be knocked out to block synthesis downstream from that point (GalNAc-T: GM2synthase).

For the experiments described in this thesis, only the first three types of mutant mice described above were used. In addition, through *in vitro* treatment of the nerve tissues with neuramindase (breaking down certain classes of gangliosides) or cholera toxin B-subunit (blocking GM1 ganglioside) it was possible to even further manipulate the ganglioside expression profile in these mice.

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 where 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.