Title: Neuropathophysiological potential of Guillain-Barré syndrome anti-ganglioside-complex antibodies at mouse motor nerve terminals

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Abstract

Objectives: Anti-ganglioside antibodies are present in about half of the patients with Guillain-Barré syndrome (GBS). Recently it has been shown that a considerable proportion of these patients has serum antibodies against antigenic epitopes formed by a complex of two different gangliosides. However, direct experimental evidence for neuropathogenicity of this special category of antibodies is currently lacking. Here we explored a series of GBS and GBS-variant sera with anti-ganglioside-complex antibodies for their ability to induce complement-dependent deleterious effects at the living neuronal membrane.

Methods: The neuropathophysiological potential of 31 GBS sera containing either anti-GM1/GD1a- or anti-GM1/GQ1b-ganglioside-complex antibodies was studied at motor nerve terminal presynaptic membranes in the mouse phrenic nerve/diaphragm muscle *ex vivo* experimental model. With electrophysiological measurements and confocal fluorescence microscopy we assessed and quantified the damaging effect on neuronal membranes by anti-ganglioside-complex antibodies.

Results: We show that anti-GM1/GD1a- and anti-GM1/GQ1b-ganglioside-complex positive sera can induce complement-mediated functional and morphological injury at mouse motor nerve terminals *ex vivo*. Of the 31 investigated anti-ganglioside-complex patient sera, 17 sera induced increases in miniature endplate potential frequency in this experimental model, mostly associated with muscle fibre twitches. Variability in potency was observed, with the anti-GM1/GD1a-complex sera inducing the most outspoken effects.

Conclusions: This study demonstrates the presence of ganglioside-complexes as available antigens in living neuronal membranes and supplies proof-of-principle that anti-ganglioside-complex antibodies in sera from GBS patients can induce complement-mediated damage. This strongly supports the hypothesis that autoimmune targeting of ganglioside-complexes is of pathogenic relevance in a proportion of GBS patients.

Keywords: antibody, ganglioside, Guillain-Barré syndrome, neuromuscular junction, pathophysiology

Introduction

Gangliosides form a family of sialic acid-containing amphiphilic glycosphingolipids that are enriched in neuronal membranes. Anti-ganglioside antibodies can be detected in around 50% of patients suffering from Guillain-Barré syndrome (GBS) or clinical variants, which are postinfectious peripheral neuropathies with diverse motor and sensory disturbances.[1-3] Anti-ganglioside antibodies are thought to exert neuropathogenic effects, either directly or through complement activation, on peripheral nerve axons including motor nerve terminals.[3-5]

It has been recently recognized that combinations of two different gangliosides can form a novel antigenic glycoepitope and that some GBS patients have antibodies against such a complex.[1,6,7] Clinical correlation and fine-specificity studies estimate that 10-20% of GBS patients has anti-ganglioside-complex antibodies.[2,8,9] Interestingly, GBS patients with anti-ganglioside-complex antibodies seem to have more severe disease symptoms than patients with antibodies against single gangliosides. In particular GBS patients (from a Japanese population) with antibodies directed against GD1a/GD1b- and GD1b/GT1b-complexes more often require mechanical ventilation.[8] However, this was not confirmed by others in an Italian GBS patient cohort.[2] In the GBS variant Miller Fisher syndrome (MFS), associated with anti-GQ1b ganglioside antibodies and characterized by ophthalmoplegia, ataxia and areflexia, the incidence of anti-complex antibodies seems higher than in GBS. One study showed that 7 of the 12 investigated MFS sera contained antibodies against a complex of at least GQ1b or GT1a and another ganglioside.[10]

The observations that anti-ganglioside-complex antibodies disappear upon clinical recovery,[9] as occurs with antibodies against single gangliosides, in combination with their association with particular disease phenotypes suggests that they play a neuropathogenic role. However, this has not yet been directly shown in experiments on living neuronal membrane. Previously, we have demonstrated that antibodies against single gangliosides can induce complement-mediated damage at neuronal membranes of motor axon terminals in mouse neuromuscular junctions (NMJs), reviewed in.[4] This effect is electrophysiologically hallmarked by a temporary extremely high frequency of miniature endplate potentials (MEPPs, the postsynaptic responses to uniquantal acetylcholine release), causing asynchronous muscle fiber twitches and, eventually, depletion of neurotransmitter which results in transmission block and thus paralyzes the muscle. Here we explored a first series of 21 GBS sera with anti-GM1/GD1a-complex antibodies and 10 GBS variant sera with anti-GM1/GQ1b-complex antibodies for their ability to induce complement-dependent deleterious effects in the mouse NMJ model system.

Methods

Patient sera and mouse monoclonal antibodies

Acute-phase serum from 31 GBS (variant) patients from Japan (26 sera), The Netherlands (4 sera) and Bangladesh (1 serum), were obtained with informed consent and local Medical Ethical Committee approval and stored until experimental use at -80°C. Normal human serum (NHS) from a healthy donor, stored in 0.5 ml aliquots at -80 °C, was used as complement source. 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.[4,11-13] Patient sera were complement-inactivated by heating at 56°C for 30 min. Sera and mAbs were dialyzed (using a 10 kD molecular weight cut-off dialysis membrane) overnight at 4°C 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), pre-gassed with 95%-O_2 / 5%-CO_2.

Enzyme-linked immunosorbent assay

Sera were tested in ELISA as described [9] for IgM and IgG antibody activity against individual gangliosides and complexes, as indicated in the Results. Serum (1:100) scoring an optical density (OD) of >0.2 was considered positive. For anti-ganglioside-complex antibodies, positivity in sera was defined as having an OD of >0.2 higher than the highest OD for antibodies against the two individual gangliosides.[9,14] All samples were tested in duplicate. Positive sera were titrated using two-fold serial dilution series starting at 1:100. The reciprocal of the highest dilution that resulted in an OD higher than the cut-off value (0.2) was taken to be the titer.

Bioassays on mouse hemidiaphragm-phrenic nerve preparations

Male and female wild-type and GD3-synthase knockout (GD3s-KO) mice [15] were used at 1-4.5 months of age. GD3s-KO mice lack the b- and c-series gangliosides due to genetic absence of GD3-synthase (Fig. 1).[16] Mice were killed with CO_2 and hemidiaphragms with phrenic nerves were dissected and kept in Ringer's medium at room temperature (20-22°C). Muscles were incubated with heat-inactivated (30 min at 56°C to destroy complement) GBS serum diluted at 33% in Ringer's medium or mouse mAbs dissolved in Ringer's for 3 h at 32ºC, rinsed in Ringer's for 10 min and exposed to 33% NHS in Ringer's for 1 h at room temperature. Micro-electrode recording of MEPPs (10-30 NMJs per session) and visual scoring of spontaneous asynchronous fiber twitching (0 for no twitching across the

hemidiaphragm, 1 for twitching of <10 fibers, 2 for a small amount, 3 for a moderate amount and 4 for an extensive amount) were done as described.[15,17] Depending on the available volumes, sera were tested 1-4 times and the mean values of the parameters were calculated. Animal experiments were carried out according to Dutch law and Leiden University guidelines, including approval of the Experimental Animal Committee of the Leiden University Medical Center.

Complement immunohistochemistry

C3c deposition at NMJs was quantified in a selection of the electrophysiologically tested samples, as described previously.[11] IgG and membrane attack complex deposition at NMJs as well as neurofilament presence at terminal motor nerve was qualitatively determined with confocal fluorescence microscopy, as described previously.[11,17]

Statistical analysis

The statistical significance of the correlation between the serum titer of antibodies against ganglioside-complexes, the mean MEPP frequency elevation a serum had induced at mouse NMJs and the C3c complement deposition values was tested for with the Spearman's rank correlation test using the VassarStats Website for Statistical Computation (http://faculty.vassar.edu/lowry/VassarStats.html), with p-values <0.05 considered as statistically significant.

Results

Anti-ganglioside-complex antibodies tests

On the basis of the ELISA results, sera were classified into two categories (Table 1): 1) anti-GM1/GD1a-complex positive (21 sera: #1-21) and 2) anti-GM1/GQ1b-complex positive (10 sera: #22-31), as determined by the centre of origin. In view of the possible inter-laboratory variation in anti-ganglioside antibody assays, [18] all sera were re-tested in ELISA for IgG and IgM antibodies against gangliosides GM1, GQ1b, GD1a and GD1b and ganglioside complexes GM1/GQ1b, GM1/GD1a, GM1/GD1b, GD1a/GD1b. Anti-ganglioside-complex positivity was confirmed in all but two sera (#22 and #29), which were negative for anti-GM1/GQ1b-complex. They were still included because they had been defined (low) positive when tested in Japan. Many sera (19/31) showed additional activity against one or both of the individual gangliosides of the complex, but generally these titers were only very low and always much lower than the titer of the anti-complex antibodies (Table 1). We reviewed the clinical neurophysiological data (mostly from arm muscle and nerve) from the patients whose serum was tested in the present study (Table 1). From 20 of the 21 anti-GM1/GD1a-complex positive patients data was available. The majority (15/20) had a reduced compound muscle action potential amplitude. Distal motor latency was normal in most patients (13/20) but was increased in 7 of the 20 patients. From 6 of the 10 anti-GM1/GQ1b-complex positive patients the neurophysiological data was available and was all normal, except for a reduced compound muscle action potential of patient #30.

Pathophysiological effects at mouse NMJs

The antigenic ganglioside density on neuronal membranes is an important factor in the pathogenicity of anti-ganglioside antibodies.[12] To optimize our experimental model for anti-GM1/GD1a-complex antibodies we used diaphragm muscles of GD3s-KO mice.[16] These mice genetically lack GD3-synthase and are therefore unable to synthesize b- and c-series gangliosides (Fig. 1). There is direct biochemical proof that these mice upregulate the membrane density of a-series gangliosides GM1 and GD1a in the brain,[16] and we have previously shown indirectly with electrophysiological and fluorescence microscopical methods using anti-GD1a and anti-GM1 mAbs that this is also the case at motor nerve terminal membrane at the NMJ.[11,12] At GD3s-KO NMJs, 12 of the 21 anti-GM1/GD1a-complex sera induced elevations of MEPP frequency (i.e. >2.4 /s, twice the control mean) during the NHS incubation (range 3.5-58.9 /s; pooled control mean before incubations was 1.2 /s; Fig. 2A, Fig. 3A, Table 1). The elevated MEPP frequencies correlated positively and in

a statistically highly significant way with the titer of the anti-GM1/GD1a-complex antibodies ($p<0.01$, $r=0.65$, Spearman's rank correlation test, Fig. 4). Such a positive correlation was not observed between elevated MEPP frequency and the titer of possible additionally present antibodies against single gangliosides GM1 ($p=0.196$, $r=0.30$) or GD1a ($p=0.07$, $r=0.4$). From 10 of the 21 sera, sufficient serum was available to be also studied at wild-type NMJs: only two of those sera (#2 and #11) induced (moderate) elevation of MEPP frequency (to 4.6 and 3.2 /s, respectively, Table 1). With 5 of the 10 anti-GM1/GQ1b-complex sera, moderately elevated MEPP frequencies were observed (range 3.3-7.2 /s) at wild-type NMJs, without correlation with anti-GM1/GQ1b-complex titer ($p=0.74$, $r=0.12$, Spearman's rank correlation test). No effect of these sera was observed on MEPP frequency at GD3s-KO NMJs (Fig. 2A, Table 1), which was as expected because the neuronal membranes of these mutant mice lack ganglioside GQ1b (Fig. 1).

The elevated MEPP frequency induced by anti-ganglioside-complex sera was generally accompanied by irregular twitching of individual muscle fibers throughout the preparation, (Fig. 2B, Table 1). Such twitches are most likely caused by superimposed MEPPs crossing the firing threshold of muscle fibers and have also been observed in our previous studies on the pathophysiological effects of sera positive for antibodies against single gangliosides and of mouse monoclonal antibodies against single gangsliosides.[17,19] Mean twitching score was <0.5 in control (pre-incubation) sessions and was similarly low with anti-GM1/GD1a-complex sera tested in wild-type muscle (range 0.0-0.6). In GD3s-KO muscles, 11 of these 21 sera scored >1.0 (range 1.0-2.8, Fig. 2B). Seven of the 10 investigated anti-GM1/GQ1b-complex sera scored >1.0 in wild-type muscles (range 1.2-2.3, Fig. 2B). At GD3s-KO muscles, two of these sera scored >1.0 (1.7 and 1.9). Mean positive control mAb score was >2.1, i.e. at GD3s-KO NMJs the anti-GM1 mAb DG2 scored 2.9 and the anti-GD1a mAb MOG35 scored 2.2, while at wild-type NMJs the anti-GQ1b mAb CGM3 scored 2.3.

Complement deposition at the NMJ

In our previous studies on antibodies against single gangliosides we have demonstrated that these can induce complement activation, culminating in membrane attack complex formation at the presynaptic nerve terminal and that this is underlying the observed temporary dramatic increase of MEPP frequency at the NMJ due to the excessive influx of Ca^{2+} through membrane attack complex pores in the presynaptic neuronal membrane.[17,20,21] We investigated here with confocal fluorescence microscopy whether anti-ganglioside-complex antibody-containing sera also induced complement activation at the NMJ. For anti-

GM1/GD1a-complex sera, complement C3c deposition at NMJs associated with elevated MEPP frequency ($p<0.01$, Spearman's rank correlation test, Table 1, Fig. 3B). IgG and membrane attack complex deposition was observed at these NMJs, as well as neurofilament loss, indicating terminal motor axonal damage (Fig. 3B), as shown previously for antibodies against single gangliosides.[22] For anti-GM1/GQ1b-complex sera, C3c deposition at NMJs was sparse and not consistently associated with MEPP frequency elevation (Table 1).

Discussion

We here report that sera positive for either anti-GM1/GD1a-complex or anti-GM1/GQ1b-complex antibodies clearly can produce pathophysiological effects at presynaptic neuronal membranes at NMJs in the mouse diaphragm/phrenic nerve *ex vivo* experimental model. Roughly half of the 31 anti-ganglioside-complex sera tested in the current experiments induced the effects, which were similar to those observed earlier with antibodies and sera with activity against either single gangliosides GQ1b, GD1a, GM1 or GD1b.[4,11,12,21] In the set of 21 anti-GM1/GD1a-complex positive sera, we found a statistically highly significant correlation between the MEPP frequency elevation observed in the electrophysiological experiments and the titer of this specific anti-complex antibody in the sera and, furthermore, an association with complement activation as quantified in fluorescence microscopical analyses. In previous studies using sera or antibodies against single gangliosides we showed that the utmost consequence at the mouse NMJ is block of evoked ACh release due to presynaptic focal complement-mediated lysis, leading to muscle paralysis. [4] Although we did not structurally monitor in the present studies whether or not anti-ganglioside complex sera induced these endpoint effects, some of them certainly caused (partial) block of the diaphragm muscle contraction evoked by nerve stimulation, as judged visually. However, especially with the sera that only induced moderate increases in MEPP frequency it is to be expected that they would not, or only after periods much longer than the current observation period of 1 h, lead to transmission block.

Thus, we here for the first time demonstrate that anti-ganglioside-complex antibodies are capable of binding to living neuronal membranes and, by activating complement, can induce pathophysiological effects. These antibodies are therefore likely of pathogenic relevance, as also suggested from the clinical association with specific patterns of paralysis and, in some patient groups, mechanical ventilation.[2,8] In a previous study, anti-GM1/GD1a-complex serum positivity was associated with a pure motor variant of GBS without severe cranial nerve involvement,[9] suggesting a specific effect of these antibodies on motor axons. Our finding of deleterious effects of anti-ganglioside-complex antibodies at mouse motor nerve terminals suggests that these antibodies may, apart from causing motor axonal dysfunction, induce some degree of NMJ synaptopathy in GBS patients, potentially contributing to the paralytic symptoms.

Due to the limited availability of most of the patient sera for repetitive experimental study and because of the heterogeneity of the anti-ganglioside(-complex) characteristics of the studied sera, some complexities of our results remain unresolved. First, not all sera induced

the deleterious effects at mouse NMJs. Second, some of the active sera, especially those from the anti-GM1/GQ1b-complex positive series, caused only moderate effects, i.e. the MEPP frequency remained lower than 10 /s, as compared to values of >20 MEPPs /s induced by the positive control mAbs and many of the active anti-GM1/GD1a-complex sera. These differences may relate to the titer and affinity variations of anti-ganglioside-complex antibodies amongst sera, together with the likely existence of an antibody binding threshold for the induction of pathophysiological effects at NMJs. Indeed, pathophysiological inactive or less active sera generally had low anti-ganglioside-complex titer and, at least in the anti-GM1/GD1a-complex series, statistical analysis showed a clear correlation between the elevated MEPP frequency and antibody titer. Third, many active sera, especially the anti-GM1/GD1a-complex positive ones, contained additional activities against single gangliosides GM1 and/or GD1a (Table 1), which in principle may have contributed to the effects. However, these single ganglioside antibody titers were generally (very) low, both in absolute sense as well as relative to the titers of the anti-GM1/GD1a-complex antibody in these sera. Furthermore there was no statistically significant correlation between the titer of anti-GM1 or anti-GD1a antibodies and the elevated MEPP frequency observed with the sera. Still, this co-presence of anti-single-ganglioside antibodies complicates interpretation, in particular because four anti-GM1/GD1a-complex sera without additional activity against GM1 or GD1a lacked effects. This could be due to their only low-positive anti-GM1/GD1a-complex titers but this may also suggest that besides anti-GM1/GD1a-complex activity, some additional anti-glycolipid or anti-glycolipid-complex activity is required for the neuropathophysiological effects. Any potential co-presence of anti-GQ1b single-ganglioside antibody in the anti-GM1/GD1a-complex sera active at GD3s-KO NMJs could not have been of influence because GQ1b ganglioside is not expressed in the plasma membranes of GD3s-KO mice (Fig. 1). The strongest direct evidence for a neuropathophysiological effect of anti-GM1/GD1a-complex antibody came from the study of serum #5. The anti-GM1/GD1a-complex antibodies in this serum unambiguously were solely responsible for the complement-mediated neuropathophysiological effects at GD3s-KO NMJs because this serum contained no additional anti-GD1a or -GM1 single-ganglioside antibodies. Some activity against GD1b ganglioside and GD1b- and GQ1b-containing ganglioside complexes was detected in this serum (data not shown) but this was irrelevant because GD3s-KO tissue lacks the b-series gangliosides GD1b and GQ1b (Fig. 1). Thus, the results obtained with this particular serum clearly provide proof-of-principle that GBS anti-ganglioside-complex antibodies can induce neuropathophysiological effects at living neuronal membranes.

Activity against the single gangliosides GM1 or GQ1b was less of a confounding factor in the GM1/GQ1b-complex group where 7 of the 10 sera lacked single ganglioside antibodies. However, only three of those induced MEPP frequency elevations at wild-type NMJs and these effects were only rather modest in magnitude. In addition, complement deposition and muscle fibre twitching did not very well correlate with elevation of MEPP frequency. No statistically significant correlation was found between the anti-GM1/GQ1b-complex antibody titer of the sera in this series and the MEPP frequency that was observed at wild-type NMJs. This indicates that anti-GM1/GQ1b antibodies generally only induce relatively weak neuropathogenic effects in this experimental model.

Anti-GM1/GD1a-complex sera induced either no neuropathophysiological effects or much less intense effects at wild-type NMJs, as compared to GD3s-KO NMJs. This indicates the requirement of an elevated anti-GM1/GD1a-complex antigen density for anti-GM1/GD1a-complex antibodies to become neuropathogenic, because the a-series gangliosides GM1 and GD1a are upregulated in neuronal membranes of GD3s-KO mice, including motor nerve terminals.[12,16] The ganglioside and ganglioside-complex expression pattern in different peripheral nerve membrane domains may vary considerably, both within and between species. It is even possible that certain GBS patients express particular predisposing ganglioside-complex densities or configurations. Development of high-affinity mouse mAbs specific for ganglioside-complexes will be an essential next step, allowing more detailed experimental studies in which these issues can be explored.

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Disclosure

None of the authors have financial interest in publication of the contents of this manuscript or have a relationship with any company that would have financial interest in publication.

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Figure legends

Figure 1. Ganglioside synthesis scheme

Ganglioside nomenclature is according to Svennerholm.[23] Membranes of wild-type mice contain all indicated gangliosides. GD3s-KO mice lack b- and c-series gangliosides (grey rectangle), due to absence of GD3-synthase. Cer = ceramide, GluCer = glucosylceramide, LacCer = lactosylceramide.

Figure 2. Overview of the neuropathophysiological effects of the investigated anti-ganglioside-complex sera

(A) Effect of anti-ganglioside-complex sera on MEPP frequency at wild-type (left panel) and GD3s-KO (right panel) mouse diaphragm NMJs. Twelve of the 21 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 (i.e. 2.4 /s, dashed line). Only two of the 10 anti-GM1/GD1a-complex sera that could be tested at wild-type NMJs (modestly) increased MEPP frequency. 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 wild-type NMJs. The anti-GQ1b mAb CGM3 was used as a positive control in wild-type tissue; anti-GD1a mAb MOG35 and anti-GM1 mAb DG2 were used as positive controls in GD3s-KO tissue. (B) Effect of anti-ganglioside-complex sera on muscle fiber twitches at wild-type (left panel) and GD3s-KO (right panel) mouse NMJs. Most anti-ganglioside-complex sera that induced MEPP frequency elevation scored higher than 1 (dashed line) for muscle fiber twitching upon visual inspection.

(Scoring: 0= no twitching, 1= twitching of <10 fibers, 2= a small amount, 3= a moderate amount and 4= an extensive amount of fibers)

Figure 3. Examples of electrophysiological and morphological effects of anti-ganglioside-complex sera on the mouse motor nerve terminal experimental model

(A) Examples of MEPPs recorded at a GD3s-KO mouse diaphragm NMJs before incubation with complement-inactivated anti-GM1/GD1a-complex serum #8 (upper trace) and during subsequent incubation of the serum pre-incubated muscle with normal human serum as complement source (lower trace). Sweep length is 10 s. (B) Examples of C3c, membrane-attack complex (MAC), IgG and neurofilament immunostaining at diaphragm NMJs (delineated by fluorescently labeled α-bungarotoxin binding to acetylcholine receptors, left

column panels). Muscle preparations had been exposed to complement-inactivated anti-GM1/GD1a-complex serum #2 (first row), serum #5 (second and third row) or complement-inactivated normal human serum (as negative control, fourth row) and were all subsequently exposed to normal human serum as complement source. IgG and C3c deposition and associated neurofilament loss are shown at anti-GM1/GD1a-complex sera-treated NMJs.

Figure 4. Positive correlation between anti-GM1/GD1a-complex titer of sera and the MEPP frequency induced at mouse NMJs

Plot graph of the average MEPP frequency at NMJs of diaphragm muscles from GD3s-KO mice induced by anti-GM1/GD1a-complex-positive GBS sera against the titer of the anti-GM1/GD1a-complex antibodies. Spearman's rank correlation test showed a highly significant positive correlation ($r=0.65$, $p<0.01$).

	serum#	patient neurophysiology		anti-ganglioside titer			GD3s-KO mouse tissue			wild-type mouse tissue		
							pathophysiology		C3c staining	pathophysiology		C3c staining
		CMAP	DML				fMEPP (/s)	twitching		fMEPP (/s)	twitching	
anti-GM1/GD1a-complex sera				**GM1/GD1a complex**	**GM1**	**GD1a**						
	1	=	=	G-25600	G-800	G-6400	58.9	+	++	1.6	-	-
	2	↓	=	G-12800	G-400	G-1600	49.3	++	+	3.2	-	-
	3	↓	=	G-51200	G-400	G-800	45.0	++	+	nt	nt	nt
	4	↓	=	G-12800	G-400	G-400	36.8	++	+++	0.5	-	nt
	5	↓	↑	G-6400	-	-	28.8	++	++	nt	nt	nt
	6	=	=	G-6400	-	G-100	27.0	++	+++	0.6	-	nt
	7	↓	=	G-12800	G-1600	G-100	19.8	++	+++	0.7	-	nt
	8	↓	↑	G-12800	G-1600	G-100	16.2	++	++	nt	nt	nt
	9	↓	↑	G-6400	G-100	G-100	14.2	++	++	nt	nt	nt
	10	↓	=	G-6400	G-100	-	6.6	-	nt	1.7	-	+
	11	↓	=	G-12800	-	G-200	4.9	+	nt	4.6	-	++
	12	↓	↑	G-400	M-200	-	3.5	-	nt	nt	nt	nt
	13	=	↑	G-6400	G-100	-	2.1	-	nt	0.5	-	nt
	14	nd	nd	G-200	-	-	2.0	-	+	nt	nt	nt
	15	↓	↑	G-400	-	-	1.7	-	+	nt	nt	nt
	16	↓	=	G-6400	-	-	1.6	-	nt	nt	nt	nt
	17	=	=	G-400	-	-	1.4	+	nt	nt	nt	nt
	18	=	=	G-3200	G-200	-	1.3	-	+	nt	nt	nt
	19	↓	=	G-12800	G-100	G-800	1.1	-	nt	1.5	-	+++
	20	↓	↑	G-1600	G-200	G-100	1.1	-	nt	2.0	-	nt
	21	↓	=	G-3200	G-800	G-400	1.1	-	-	nt	nt	nt
anti-GM1/GQ1b-complex sera				**GM1/GQ1b complex**	**GM1**	**GQ1b**						
	22	=	=	- †	-	-	1.6	-	+	7.2	-	+
	23	=	=	G-1600	-	G-400	1.8	-	+	6.0	+	++
	24	nd	nd	G-800	-	-	1.4	-	+	4.6	++	+
	25	nd	nd	G-800	-	-	2.1	+	nt	4.3	+	nt
	26	nd	nd	G-3200	-	G-400	1.5	-	nt	3.3	+	nt
	27	=	=	G-1600	-	-	1.9	-	nt	2.3	+	nt
	28	nd	nd	G-400	-	-	2.3	-	nt	2.3	-	nt
	29	=	=	- †	M-100	-	1.3	+	nt	1.7	-	nt
	30	↓	=	G-400	-	-	1.7	-	nt	1.6	+	nt
	31	=	=	G-800	-	-	1.2	-	nt	1.6	+	nt

Table 1. Patient neurophysiology, serum anti-ganglioside characteristics and neuropathogenicity of the investigated sera.
Patient neurophysiology, serum anti-ganglioside antibody activity (either IgG (G) or IgM (M)), effects on MEPP frequency (fMEPP), muscle fiber twitching and C3c deposition at neuromuscular junctions of sera positive for either anti-GM1/GD1a antibodies (sera #1-21) or anti-GM1/GQ1b antibodies (sera #22-31). The sera have been ranked in descending order according to the observed MEPP frequency (for anti-GM1/GD1a-complex sera in GD3s-KO tissue and for anti-GM1/GQ1b-complex sera in wild-type tissue).

Anti-ganglioside titer: - = negative

Twitching: - = <1.0; + = between 1.0 and 2.0; ++ = >2.0.

C3c staining was performed in two comparable runs on 17 and 7 tissue samples. Indicated is the relative intensity within these series: + = low; ++ = moderate; +++ = high.

† these sera were tested positive for anti-GM1/GQ1b-complex in the centre of origin

CMAP = compound muscle action potential
DML = distal motor latency
nt = not tested
nd = no data available

Figure 1

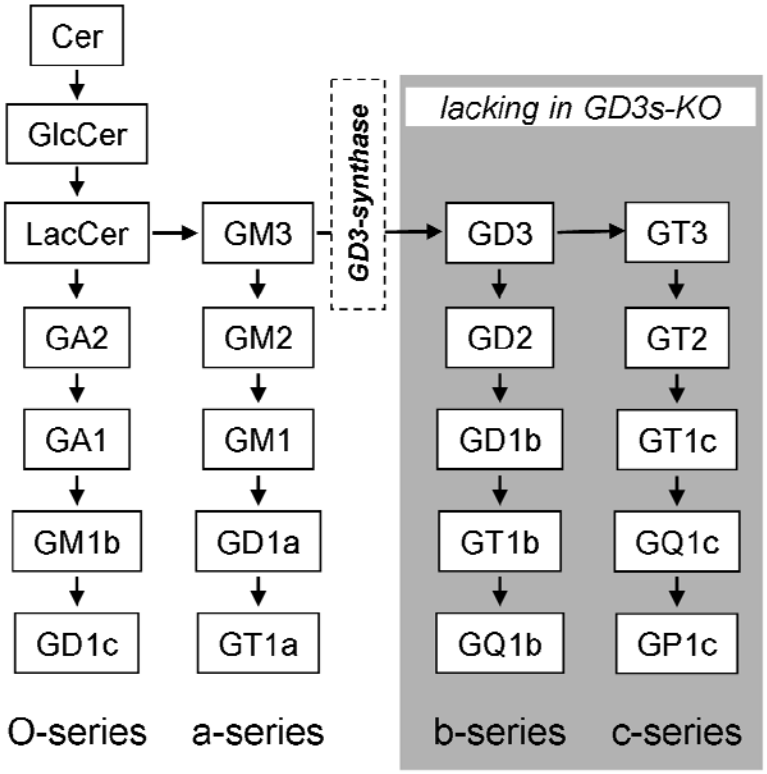

Cer
GlcCer
LacCer
GA2
GA1
GM1b
GD1c
GM3
GM2
GM1
GD1a
GT1a
GD3-synthase
lacking in GD3s-KO
GD3
GD2
GD1b
GT1b
GQ1b
GT3
GT2
GT1c
GQ1c
GP1c
O-series
a-series
b-series
c-series

Figure 2

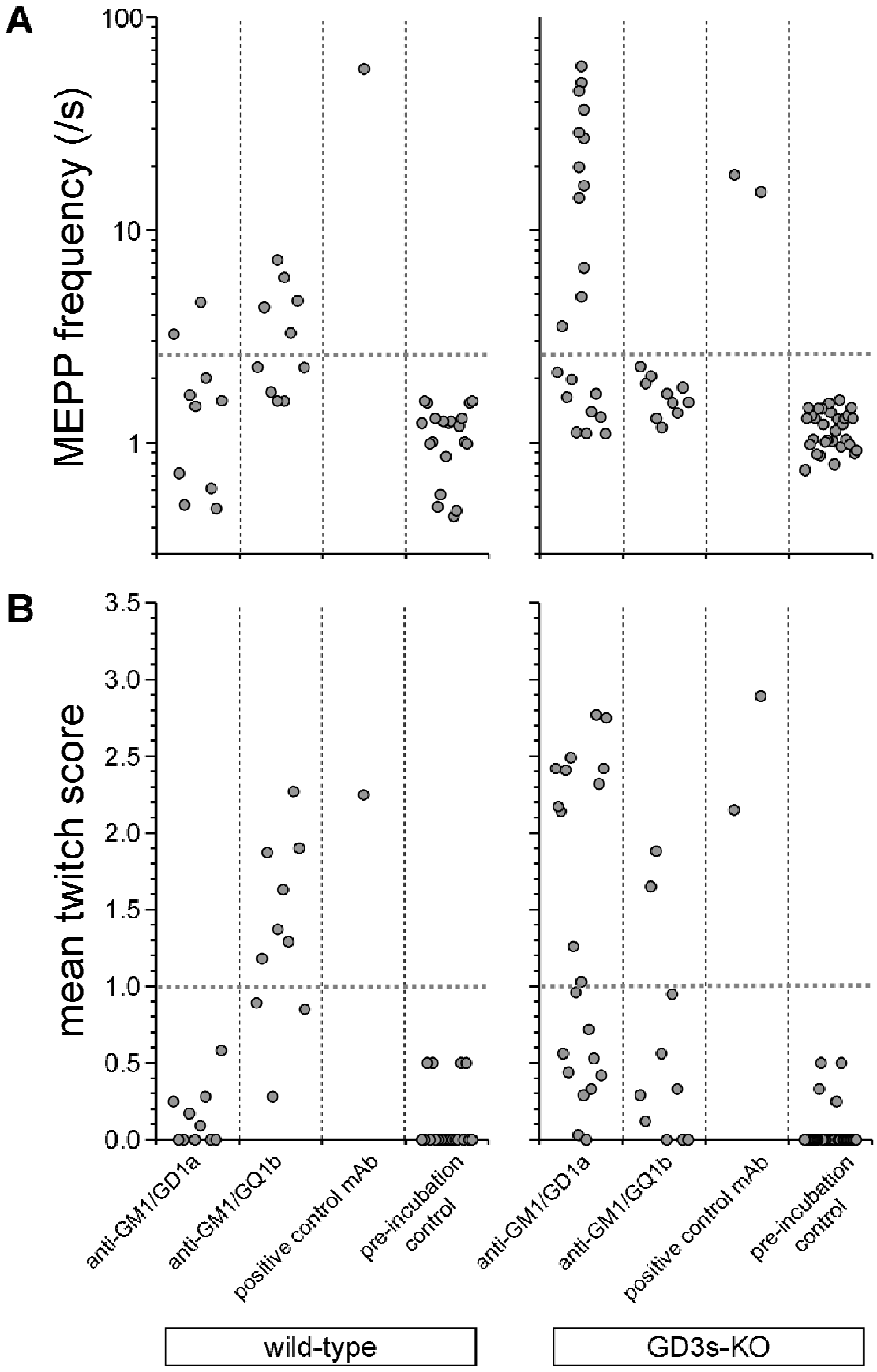

A
MEPP frequency (/s)
100
10
1
B
mean twitch score
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
anti-GM1/GD1a
anti-GM1/GQ1b
positive control mAb
pre-incubation control
anti-GM1/GD1a
anti-GM1/GQ1b
positive control mAb
pre-incubation control
wild-type
GD3s-KO

Figure 3

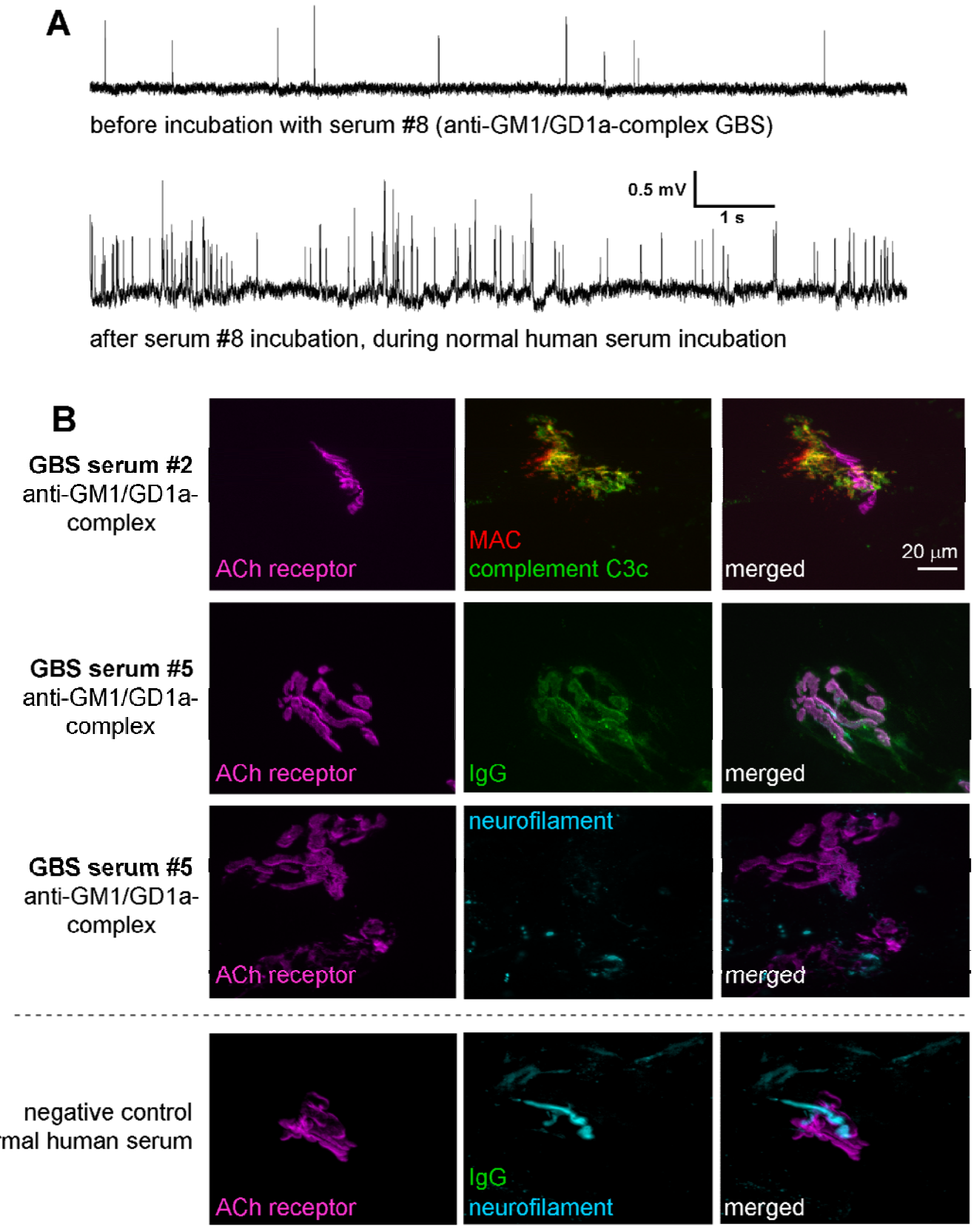
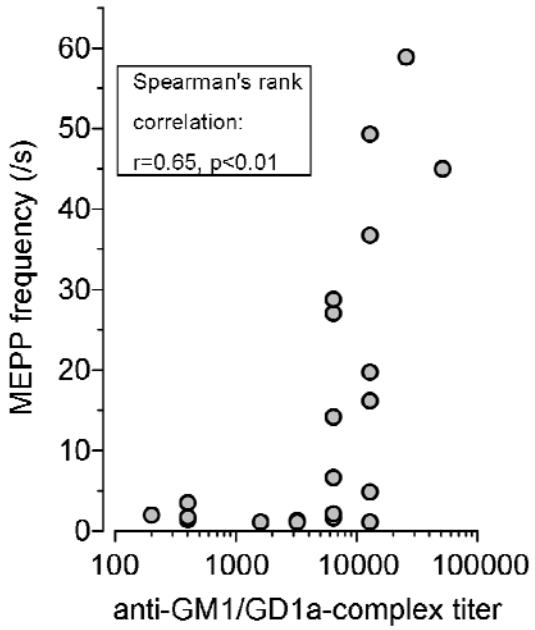

A
before incubation with serum #8 (anti-GM1/GD1a-complex GBS)
0.5 mV
1 s
after serum #8 incubation, during normal human serum incubation
B
GBS serum #2
anti-GM1/GD1a-complex
ACh receptor
MAC
complement C3c
merged
20 µm
GBS serum #5
anti-GM1/GD1a-complex
ACh receptor
IgG
merged
GBS serum #5
anti-GM1/GD1a-complex
ACh receptor
neurofilament
merged
negative control
normal human serum
ACh receptor
IgG
neurofilament
merged

Figure 4


Spearman's rank
correlation:
r=0.65, p<0.01
MEPP frequency (/s)
60
50
40
30
20
10
0
100
1000
10000
100000
anti-GM1/GD1a-complex titer