Gangliosides and anti-ganglioside antibodies in neuromuscular synaptic function

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CHAPTER 3.3

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

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Abstract

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

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Introduction

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

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

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

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

Methods

Mice

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

Antibodies, sera and protein

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

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

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

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

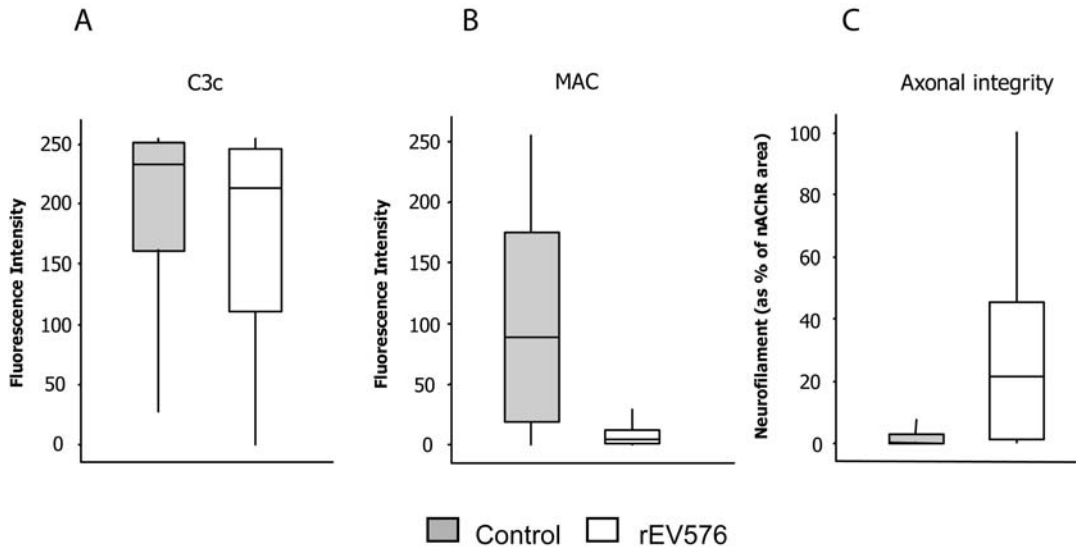

Figure 3.12. Mouse hemi-diaphragm *in vitro* preparations incubated with anti-GQ1b mAb CGM3 followed by NHS with added rEV576 or BSA, as control protein, at 100 µg/ml. The staining intensity of (**A**.) complement C3c was slightly reduced and of (**B.)** membrane attack complex (MAC) was substantially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

Immunohistology

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

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

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

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

Image acquisition, quantitation and statistical analysis

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

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

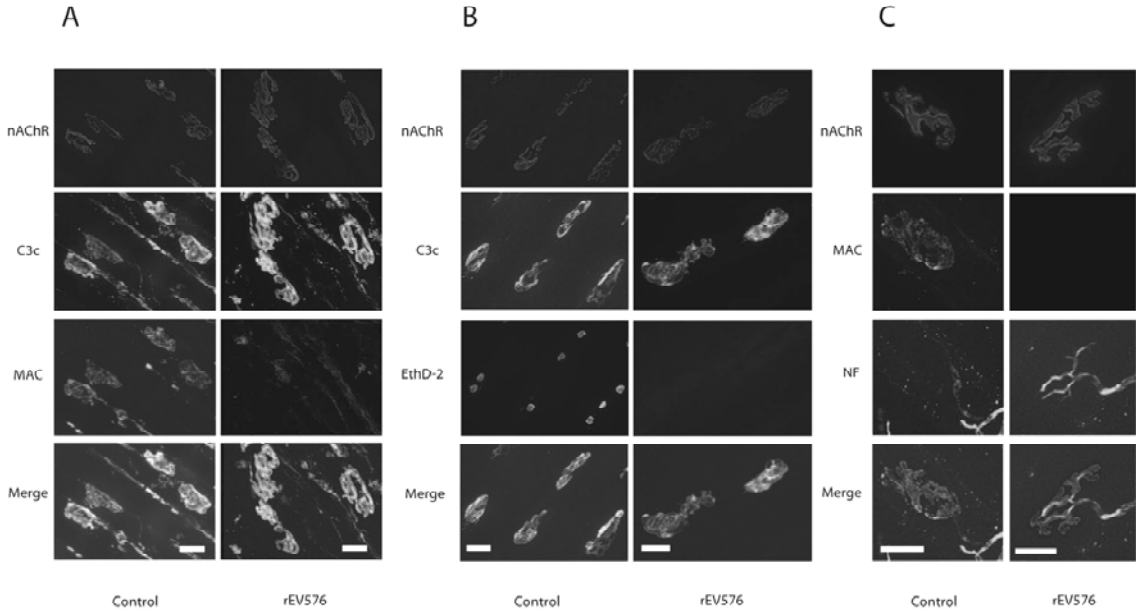

Figure 3.13. Illustrative immunofluorescent images of whole-mount muscles demonstrating neuronal and perisynaptic Schwann cell (pSC) protection at neuromuscular junctions (NMJs) by rEV576: In all panels, postsynaptic nicotinic acetylcholine receptors (nAChR) staining was used to delineate the NMJ. **A.**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

In vitro electrophysiology

Hemi-diaphragms were dissected with the phrenic nerve attached and pinned out on a silicone rubber-lined dish in Ringer's medium pre-gassed with 95% O_2 / 5% CO_2 at RT. Samples were incubated for 2.5 h with 50 µg/ml anti-GQ1b ganglioside CGM3 at 32 °C. Subsequently the preparations were rinsed in Ringer's and then treated for 1 h at RT with 33% NHS with added rEV576 or control protein BSA at 100 µg/ml, pre-mixed 10 min before application. Using a glass microelectrode (10-20 MΩ) filled with 3 M KCl, intracellular recordings of miniature endplate potentials (MEPPs), arising from presynaptic spontaneous uniquantal release of acetylcholine (ACh), were made at NMJs of preparations at RT just before the CGM3 incubation and again during the 1 h rEV576 or BSA incubation period. Recordings were amplified and filtered (10 kHz low-pass, 1 Hz high-pass) with a GeneClamp 500B amplifier and digitized and stored using a Digidata 1322A interface, Clampex 9.2 and Clampfit 9.2 (all from Axon Instruments/Molecular Devices). Data was analyzed off-line with mini-analysis 6.0 (Synaptosoft).

Visual scoring of muscle fibre twitchings and evoked muscle contraction
During the electrophysiological assessment of the hemi-diaphragms incubated with NHS and rEV576/BSA, the muscles were visually scored (grades 0-4) for the occurrence of spontaneous asynchronous fibre twitching, as described before (O'Hanlon et al., 2001). These twitches are a hallmark of CGM3/complement-mediated destruction of NMJs (Plomp et al., 1999). We also scored (grades 0-2) for the extent of tetanic muscle contraction upon 40 Hz nerve stimulation. In addition, spontaneous asynchronous twitching was visually scored (0-4) in an assay using small diaphragm strips, as described before (Jacobs et al., 2002). The incubation protocol was as described above for the complete hemi-diaphragms. Scores were summed over time. After the NHS and rEV576/BSA incubation period the strips were assessed electrophysiologically for the percentage of "silent" NMJs (i.e. without any detectable synaptic electrophysiological signal), which is the final result of the presynaptic damage caused by complement.
Group comparisons were made using a Students *t*-test. A p value <0.05 was considered to be significant.

Results

Immunohistology
The C5 inhibitory effects of rEV576 were tested in an *in vitro* mouse model of MFS. Mouse hemi-diaphragm preparations were incubated with monoclonal anti-ganglioside antibody CGM3, followed by NHS with added rEV576 or control protein BSA.
C3c intensity at the NMJ (Figure 3.12A) was reduced by a small but statistically significant degree in hemi-diaphragm tissue treated with rEV576 (median level, 214; n=746) compared with control (median level, 233; n=727; $p<0.01$), despite similar intensities of IgM deposits (data not shown). In contrast to C3c levels and as predicted, MAC intensity (Figure 3.12B) was greatly reduced in tissue treated with rEV576 (median level, 5; n=678), compared to control tissue (median level, 88; n=704; $p<0.01$). Axonal integrity was assessed by NF staining over the NMJ (Figure 3.12C), which was abolished in control tissue (n=678) but preserved in rEV576-treated tissue (n=704). EthD-2 uptake was extensive, as expected, in tissue treated with CGM3 and NHS plus BSA control protein (54% of NMJs showed positive nuclei; n=2338), while only 1% of NMJs (total sampled n=1954) demonstrated damaged pSC in rEV576-protected tissue (chi square test, $p<0.01$).
Illustrative immunofluorescent images of whole-mount muscles demonstrating neuronal and pSC protection at NMJs by rEV576 are shown in Figure 3.13. In all upper panels, postsynaptic nAChRs were stained to delineate the NMJ, and this postsynaptic staining remains unaffected throughout, as shown in previous studies. In the presence or absence of rEV576, intermediate complement cleavage product C3c deposits were seen in tissues treated with anti-ganglioside antibody plus human complement (Figure 3.13A, B). However, no MAC was seen overlying the NMJ in tissue treated with rEV576 (Figure 3.13A, C). pSC damage as manifested by EthD-2 uptake into pSC nuclei, occurred in control-treated tissue but was absent in tissue treated with rEV576, as shown in Figure 3.13B. Axonal integrity was compromised in control tissue exposed to anti-ganglioside antibody plus NHS, as demonstrated by the

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

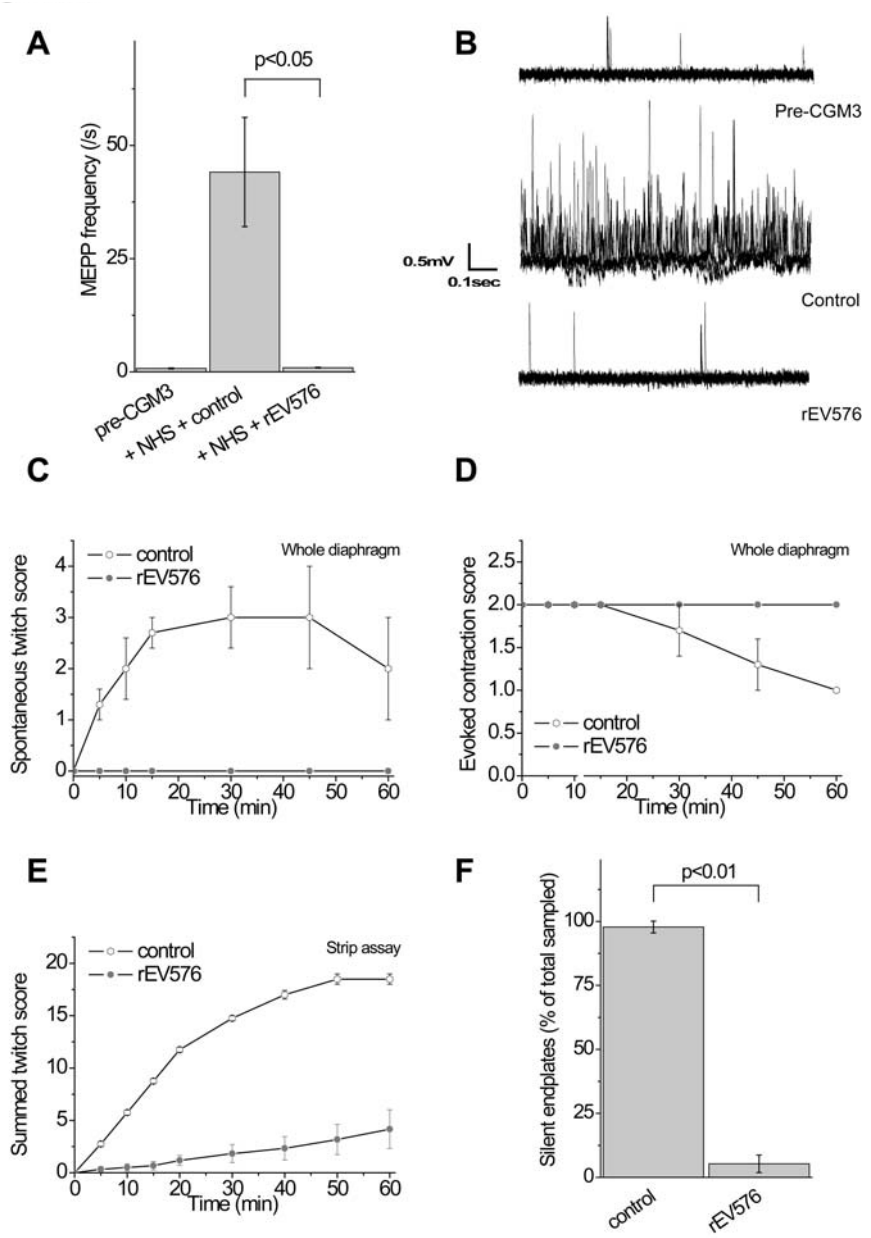

Figure 3.14. Mouse hemi-diaphragm preparations were pre-incubated with 50 µg/ml anti-GQ1b ganglioside CGM3 for 2.5 h at 32 °C. Subsequently the preparations were treated for 1 h with 33% NHS with either rEV576 or control BSA protein added at 100 µg/ml. **A.** Spontaneous uniquantal acetylcholine release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

Electrophysiology

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

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

Discussion

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

MAC is heavily deposited, most likely due to a positive feedback effect of MAC on complement activation.
The data presented here are comparable in terms of therapeutic efficacy of rEV576 to a recent study showing a substantially protective effect in an antibody and complement-mediated model of myasthenia gravis in the rat (Hepburn et al., 2007). That study also reported the known biochemical data in detail on the half-life, complement-inhibiting mechanisms and immunogenic properties of rEV576.
In our mouse model of MFS, complement is activated through the classical pathway and directed to the motor nerve terminal and pSC by membrane-bound anti-GQ1b antibody. Wallerian degeneration can result as a secondary effect after neuroimmunological axonal attack and this process has been shown to be complement dependent and delayed by complement inhibition (Ramaglia et al., 2007; Ramaglia et al., 2008). Thus, complement inhibition as a therapeutic strategy for treating peripheral nerve axonal disorders may have a dual benefit.
The field of complement therapeutics is growing rapidly with a range of agents now available for pre-clinical and clinical testing that inhibit complement activation at various stages of the cascade (Ricklin and Lambris, 2007). One particular advantage of complement inhibition at the level of C5 that rEV576 fulfils is that the antimicrobial opsonization effects of upstream complement factors are not inhibited. Conversely, this could also be a disadvantage in terms of therapeutic effectiveness when considering the possible role of macrophage and polymorphic cell infiltration in the pathogenic cascade in patients.
While this study demonstrates the therapeutic principle of axonal neuroprotection for rEV576 in a neuropathy model using an anti-GQ1b antibody directed at the motor nerve terminal, in accord with our previously published data, it is highly probable that any axonal membrane localized elsewhere and sensitized to complement attack by any other anti-ganglioside antibody would be similarly protected. Our previous work has shown complete concordance between mouse and human anti-GQ1b and other ganglioside IgG and IgM antibodies with respect to their MAC-dependent paralytic effects at the motor nerve terminal. These data should thus give further impetus towards clinical trialling of complement inhibitors in human autoimmune neuropathies.