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The Effects of Age and Ganglioside Composition on the Rate of Motor Nerve Terminal Regeneration Following Antibody-Mediated Injury in Mice

ANGIE RUPP,[1‡] MADELEINE E. CUNNINGHAM,[1‡] DENGGAO YAO,[1] KOICHI FURUKAWA,[2] AND HUGH J. WILLISON[1]*

[1]*Neuroimmunology Group, Institute of Infection, Immunity and Inflammation, College of Medical, Veterinary and Life Sciences, Glasgow Biomedical Research Centre, University of Glasgow, Glasgow G12 8TA, United Kingdom*
[2]*Department of Biochemistry II, Nagoya University Graduate School of Medicine, Showa-ku, Nagoya 466-0065, Japan*

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ABSTRACT Gangliosides are glycosphingolipids highly enriched in neural plasma membranes, where they mediate a diverse range of functions and can act as targets for auto-antibodies present in human immune-mediated neuropathy sera. The ensuing autoimmune injury results in axonal and motor nerve terminal (mNT) degeneration. Both aging and ganglioside-deficiency have been linked to impaired axonal regeneration. To assess the effects of age and ganglioside expression on mNT regeneration in an autoimmune injury paradigm, anti-ganglioside antibodies and complement were applied to young adult and aged mice wildtype (WT) mice, mice deficient in either b- and c-series (GD3sKO) or mice deficient in all complex gangliosides (GM2sKO). The extent of mNT injury and regeneration was assessed immediately or after 5 days, respectively. Depending on ganglioside expression and antibody-specificity, either a selective mNT injury or a combined injury of mNTs and neuromuscular glial cells was elicited. Immediately after induction of the injury, between 1.5% and 11.8% of neuromuscular junctions (NMJs) in the young adult groups exhibited healthy mNTs. Five days later, most NMJs, regardless of age and strain, had recovered their mNTs. No significant differences could be observed between young and aged WT and GM2sKO mice; aged GD3sKO showed a mildly impaired rate of mNT regeneration when compared with their younger counterparts. Comparable rates were observed between all strains in the young and the aged mice. In summary, the rate of mNT regeneration following anti-ganglioside antibody and complement-mediated injury does not differ majorly between young adult and aged mice irrespective of the expression of particular gangliosides. **Synapse 67:382–389, 2013.** © 2013 Wiley Periodicals, Inc.

INTRODUCTION

Gangliosides are glycosphingolipids found within microdomains on cell membranes throughout the body, but enriched in neural tissue (Hamberger and Svennerholm, 1971; Yu et al., 2011). Different gangliosides are distinguished from one another by the number and location of sialic acid residues attached to a neutral sugar backbone ((Svennerholm, 1963; Yu et al., 2011); Fig. 1). The function of gangliosides is very diverse, ranging from neural development, axonal growth, signal transduction, modulation of membrane proteins, node of Ranvier stability and receptor functions, to cell–cell interactions (Plomp and Willison, 2009; Susuki et al., 2007; Zeller and Marchase, 1992). In disease-related roles, they act as targets for toxins and autoantibodies (autoAbs).

‡A.R. and M.E.C. contributed equally to this work.

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*Correspondence to: Hugh J. Willison, Glasgow Biomedical Research Centre, Room B330, 120 University Place, Glasgow G12 8TA, United Kingdom. E-mail: hugh.willison@glasgow.ac.uk

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Anti-ganglioside antibodies (Abs) are observed in patients suffering from the human peripheral nerve disorder Guillain-Barré syndrome (GBS) (Yuki and Hartung, 2012) and are believed to be pathogenic. Much research in GBS has focused on the neuromuscular junction (NMJ) as a potential site of anti-ganglioside Ab binding and injury (Halstead et al., 2005a; Jacobs et al., 2003; O'Hanlon et al., 2001; Santafe et al., 2005), as this structure lies outside the blood–nerve barrier (Olsson, 1968) and thus is exposed to blood-borne circulating factors such as autoAbs and ganglioside-binding neurotoxins. Following the binding of anti-ganglioside Abs to ganglioside-rich plasma membranes, one mechanism by which injury is induced is through activation of the complement cascade, which culminates in the formation of membrane attack complex on the structures bound (Halstead et al., 2005a). The membrane attack complex pore allows for an uncontrolled influx of ions and water and ultimately results in pathological changes and dysfunction of the structures targeted (Halstead et al., 2005b; McGonigal et al., 2010). Depending on the Ab specificity, a selective injury of the neural structures of the NMJ, the motor nerve terminals (mNTs) or the glial structures of the NMJ, the perisynaptic Schwann cell (pSCs), is elicited (Halstead et al., 2005b).

Ganglioside-deficient mice (Fig. 1), generated by glycosyltransferase knock-out (KO), are widely used in experiments investigating the effects of anti-ganglioside Abs (Goodfellow et al., 2005; Lehmann et al., 2007; Sheik et al., 2004). GM2-synthase (also known as β1,4-*N*-acetylgalactosaminyltransferase) KO mice (GM2sKO) mice are deficient in complex gangliosides and express increased levels of the simple gangliosides GM3, GD3, and GT3 (Takamiya et al., 1996). Similarly, GD3-synthase (also known as α-2,8-sialyltransferase) KO mice (GD3sKO) are deficient in b- and c-series gangliosides, whereas expressing gangliosides of the a-series at a higher level when compared with wildtype (WT) mice (Kawai et al., 2001; Okada et al., 2002). Both of these strains are viable and at young adult age do not appear to exhibit any obvious morphological or behavioral phenotypes (Kawai et al., 2001; Liu et al., 1999; Okada et al., 2002; Takamiya et al., 1996; Zitman et al., 2008). Aged GM2sKO mice (>9 months), however, show impaired motor behavior with a lack of balance, coordination and strength, and exhibit a whole body tremor (Chiavegatto et al., 2000; Sugiura et al., 2005; Zitman et al., 2011). Age-matched GD3sKO mice exhibit no overt neurological changes (Zitman et al., 2011). Peripheral nerve regeneration following axotomy is reported to be reduced in GD3sKO mice (Okada et al., 2002). General comparisons of peripheral nerve regeneration in young and aged animals have shown that even though axonal regeneration and reinnervation of target organs are maintained throughout life, there is a tendency for them to be less effective in aged animals when compared with their young counterparts (Verdú et al., 1995). This age-related decrease in the regeneration potential may reflect the fact that high age is found to be a poor prognostic factor for GBS patients (Durand et al., 2006; Rajabally and Uncini, 2012; van Koningsveld et al., 2007; Walgaard et al., 2011).

This study aimed to compare the regenerative potential of mNTs in young and aged mice following an immune-mediated injury induced by anti-ganglioside Abs and complement. To be able to also provide some information on the regenerative capacities of mNTs in ganglioside KO strains, these investigations were not only conducted in WT mice but also in GM2sKO and GD3sKO mice.

Abbreviations

Ab	antibody
CFP	cyan fluorescent protein
GBS	Guillain-Barré syndrome
GD3sKO	GD3-synthase knock-out mouse
GFP	green fluorescent protein
GM2sKO	GM2-synthase knock-out mouse
KO	knock-out
mNT	motor nerve terminal
nAChR	nicotinic acetylcholine receptor
NHS	normal human serum
NMJ	neuromuscular junction
pSC	perisynaptic Schwann cell
SH	sternohyoid muscle
WT	wildtype.

MATERIALS AND METHODS

Mice

All investigations were conducted in young adult (8–12 weeks) and aged (9–12 months) single- and double-fluorescent homozygous GD3sKO, homozygous GM2sKO, and WT mice, which expressed intracytosolic cyan fluorescent protein (CFP) in their peripheral motor and sensory axons and optionally also intracytosolic green fluorescent protein (GFP) in their Schwann cells (Feng et al., 2000; Zuo et al., 2004). To generate these mice, single- and double-fluorescent adult B6.Cg-Tg(Thy1-CFP ± S100B-GFP) [generously supplied by Dr. W. Thompson (Austin, TX) and now available commercially as individual lines through Jackson, Bar Harbor, ME] were crossed with GD3sKO (Okada et al., 2002) and GM2sKO mice (Takamiya et al., 1996) (imported to UK and bred from Furukawa stock, Nagoya, Japan). Inheritance of transgenes was confirmed by PCR and phenotyping of ear-punches. For the latter, the subcutis of the ears was examined for the occurrence of GFP in adipocytes and CFP in axons/neurons with a compound fluorescence microscope (Zeiss AxioImager (Zeiss, Goettingen, Germany)).

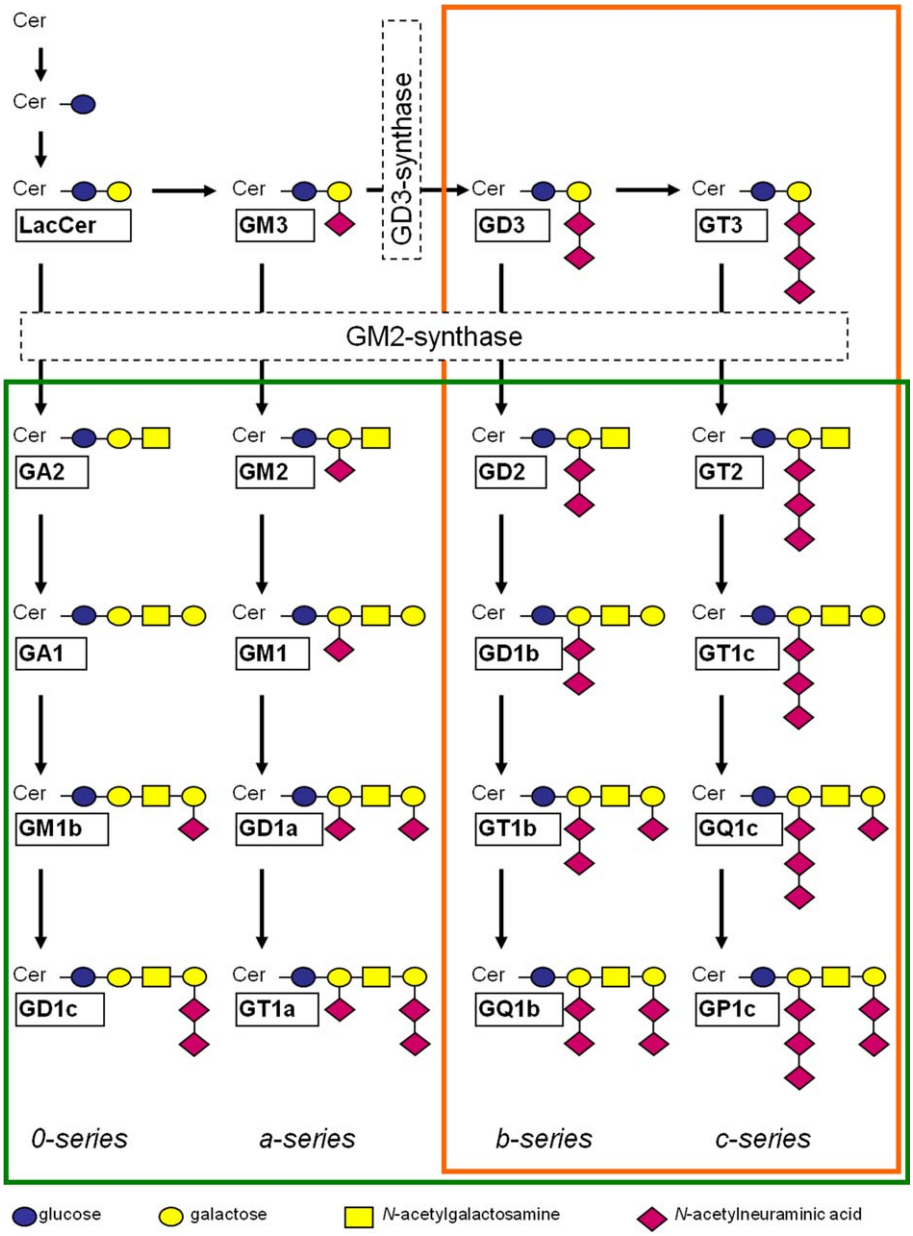

Fig. 1. Schematic overview of the synthesis and composition of gangliosides (adapted from Plomp and Willison, 2009). Mice lacking GD3-synthase (α-2,8-sialyltransferase) do not express any b- and c-series gangliosides (orange box), whereas mice lacking GM2-synthase (β1,4-*N*-acetylgalactosaminyltransferase) do not express any complex gangliosides (green box).

Animal groups

Following the in vivo induction and documentation of the anti-ganglioside Ab- and complement-mediated injury, mice were either immediately sacrificed to assess the extent of injury (d0), or recovered and assessed after 5 days (d5) to determine the extent of regeneration at this time-point ($n = 3$ per time-point and age/strain group). Young adult control mice, which only received complement and were subjected to the in vivo imaging procedures, also were assessed at d5.

Antibodies and complement

The mouse monoclonal Abs TBG3 and EG1, both IgG3 subclass, were generated and characterized for ganglioside-binding profiles as described previously (Goodfellow et al., 2005; Goodyear et al., 1999; Halstead et al., 2005b) (Table I). Both these Abs were applied topically to the muscles under investigation at absolute values of 120 μg per mouse, diluted in sterile Ringer's solution (Vetivex 9, Decra, Shrewsbury, UK).

Normal human serum (NHS), which served as a source of complement, taken from a single donor stock,

TABLE I. Antibodies applied to the different animal groups/strains, their predominant binding specificities according to ELISA, and the injury observed following the additional application of complement

Group/strain	Antibody	Antibody binding specificity	Resulting injury
WT-1	TBG3	GD1a	mNT only
WT-2	TBG3 + EG1	GD1a, GD3, GQ1b	mNT and pSC
GD3sKO	TBG3	GD1a	mNT only
GM2sKO	TBG3 + EG1	GD1a, GD3, GQ1b	mNT and pSC
Control (all strains)	None	n/a	None

was stored immediately after acquisition in aliquots at −70°C to preserve complement activity. For in vivo applications, NHS was diluted to a concentration of 40% in sterile Ringer's solution and also applied topically, with each mouse receiving a total of 0.6 mL.

In vivo procedures

All procedures were conducted in accordance with UK Home office guidelines and carried out as described previously (Rupp et al., 2012). Briefly, under general anesthesia the sternohyoid muscles (SH) of the mice were first exposed to rhodamine-conjugated α-bungarotoxin (Molecular Probes, Eugene, OR; 1:400 in sterile Ringer's solution), which selectively labels the nicotinic acetylcholine receptors (nAChRs), for 10 min. After a Ringer's wash, anti-ganglioside Abs/Ringer's solution (see Table I) were applied topically for 30 min, followed by NHS (also 30 min). In vivo imaging of NMJs ensured documentation of integrity and injury of the neuromuscular structures before and after application of Ab and complement, respectively. Originally imaged NMJs were identified due to their location and unique nAChR pattern.

Ex vivo procedures

For quantitative assessments of mNT injury and regeneration at d0 or d5, respectively, fixed SH were imaged ex vivo under coverslips with a Zeiss AxioImager compound fluorescence microscope. One hundred superficial NMJs per SH were assessed for the presence or absence of CFP overlying the NMJ. CFP was scored as "present" if uniformly intact (as opposed to fragmented) CFP extending from the efferent axon was seen overlying the NMJ, regardless of its morphology, intensity and extent. The percentage of NMJs exhibiting CFP was then calculated for each muscle assessed ($n = 2$ per mouse) and pooled for each age-group, strain, and time-point. Chi-square or Fisher's exact tests were applied to determine statistical significance. The data acquired was compared with rate of mNT injury and recovery in young WT mice presented recently (Rupp et al., 2012).

Imaging and image processing

In vivo images were acquired with an epifluorescence microscope (Leica DMI4000B), connected to a computer via a camera (Leica DFC 350 Fx, both Leica, Wetzlar, Germany) and combined with the appropriate software (Leica Application Suite Version 2.5.0 RI, Leica Microsystems CMS GmbH, Switzerland). For contrast optimization and manual reconstruction of stacks and composite images, a combination of ImageJ (Version 1.38, NIH, Bethesda, MD) and Adobe Photoshop CS (Version 8.0, Adobe Systems Europe Limited, Edinburgh, UK) were used.

Ex vivo images were acquired with a Zeiss AxioImager compound fluorescence microscope and semi-automatic Zeiss Axiovision 4.7.2 imaging software (© Carl Zeiss Imaging Solutions GmbH, Germany). This software was also used for the reconstruction of stacks and optimization (background correction) of images.

RESULTS

Anti-ganglioside Ab and complement-mediated injury

Depending on the binding specificity of the anti-ganglioside Ab applied, a selective injury to the mNTs (loss of CFP overlying the NMJ) or a combined injury to the mNTs and pSCs (loss of both CFP and GFP overlying the NMJ) was observed (Fig. 2). In WT mice, the application of TBG3 and complement was associated with a selective mNT injury (WT-1), whereas the combined application of TBG3 and EG1 lead to a combined mNT and pSC injury (WT-2). In GM2sKO mice, the application of EG1 and complement elicited a loss of both CFP and GFP, thus inducing a combined mNT and pSC injury, and in GD3sKO mice, the application of TBG3 and complement lead to a selective loss of CFP, thus inducing a selective mNT injury. The application of human serum as a source of complement (in the absence of anti-ganglioside Ab) and exposure of muscles to in vivo imaging procedures did not induce any changes to the fluorescent proteins of the NMJ (Table I; Fig. 2).

The extent of mNT injury following an anti-ganglioside Ab and complement-mediated injury differed between the various mouse strains (Fig. 3) and statistically significant differences were observed when comparing strains which had been subjected to the same type of injury (WT-1 vs. GD3sKO: $P = 0.0001$; WT-2 vs. GM2sKO: $P < 0.0001$). The average percentage of NMJs still exhibiting cytosolic CFP (i.e., intact mNT) following induction of the injury ranged from 1.5% (GD3sKO) to 11.8% (WT-2).

Motor nerve terminal regeneration

Following 5 days of regeneration, most NMJs assessed had recovered their CFP (Fig. 3). Whilst in

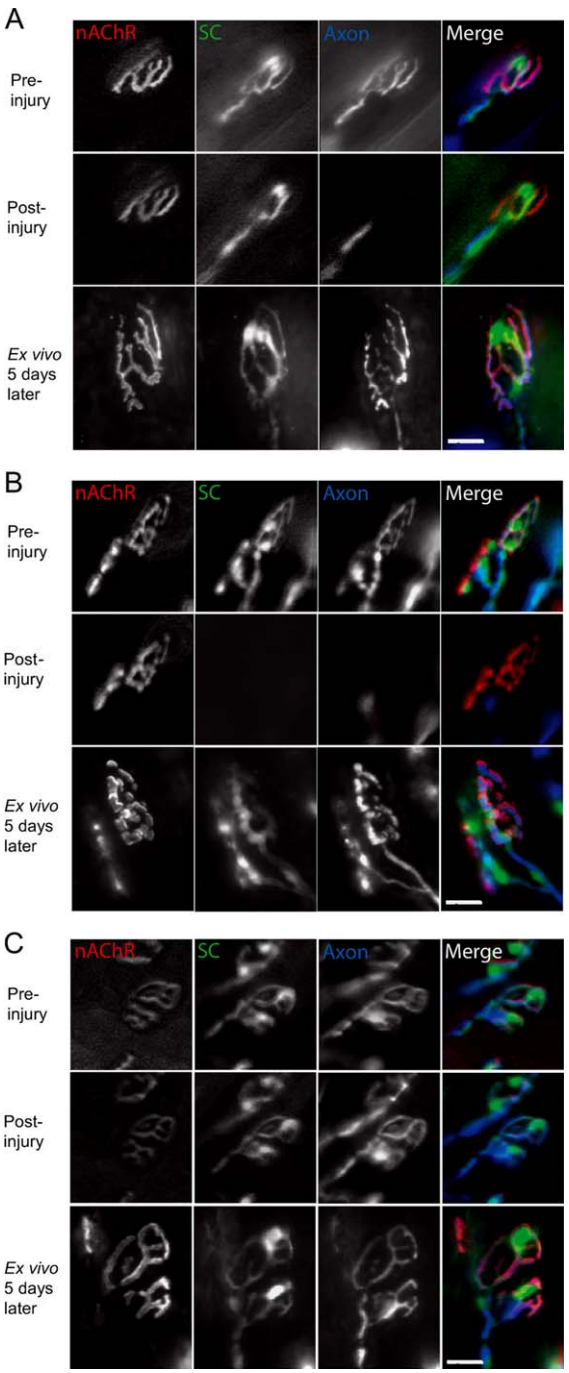

Fig. 2. Examples for injury and regeneration of the presynaptic structures in the different mouse strains. **A**: Isolated mNT injury in an aged GD3sKO mouse. **B**: Combined mNT and pSC injury in an aged GM2sKO mouse. **C**: Young control GD3sKO mouse. Note the selective loss of CFP overlying the NMJ (depicted by the nAChRs) in (A), whereas in (B) both GFP and CFP are lost following induction of the injury. After 5 days of the regeneration, both GFP and CFP are recovered, with an increased number of GFP-positive cell bodies overlying the NMJ in (B). No changes to the fluorescent proteins are noted in the control mouse (C), which only received complement, was subjected the in vivo imaging procedures and also was reimaged after a 5-day interval. Scale bars: 20 μm; pre- and post-injury images were acquired in vivo.

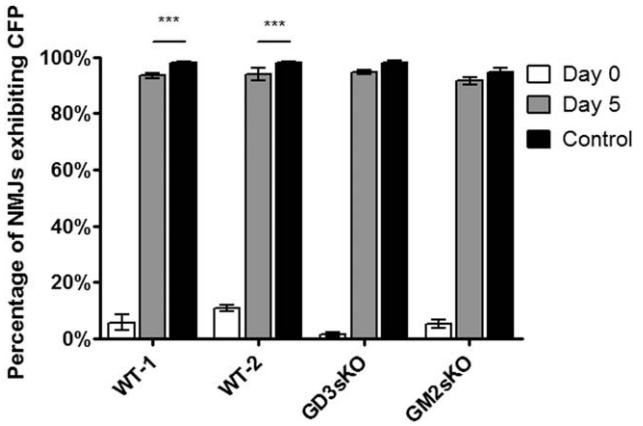

Fig. 3. Extent of mNT injury and regeneration (assessed by CFP overlying the NMJ) following application of anti-ganglioside Abs and complement in young adult WT, GD3sKO, and GM2sKO mice. Due to ganglioside expression and specificity of the anti-ganglioside Abs applied, WT-1 and GD3sKO mice experienced a selective mNT injury, whereas WT-2 and GM2sKO mice experienced a combined mNT and pSC injury. Following 5 days of regeneration, most NMJs under examination exhibited CFP and the values obtained in the experimental groups tallied very closely with their control counterparts. In the two KO strains, no statistically significant differences were observed between experimental and control tissue (GD3sKO: $P = 0.21$, Fisher's exact test, $n = 3$; GM2sKO: $P = 0.19$, Fisher's exact test, $n = 3$), whereas, in the two WT groups, statistically significant differences were observed between experimental and control tissue ($P < 0.0001$, Chi-square test, $n = 3$). Data for each group is presented as mean and standard error of the mean.

the two KO-strains no statistically significant difference was observed between the young animals undergoing regeneration and their controls (GM2sKO: $P = 0.19$; GD3sKO: $P = 0.21$), a statistically significant difference was observed between experimental and control animals in the young adult WT mice ($P < 0.0001$).

When comparing mNT regeneration in young adult versus aged mice in the various strains (Fig. 4), no statistically significant differences could be observed in the WT mice (regardless of the injury type) and the GM2sKO mice. Only the aged GD3sKO mice showed significantly impaired mNT regeneration when compared with their younger counterparts ($P = 0.0009$).

Comparison of mNT regeneration in the young age-groups with one another revealed no statistically significant difference between both groups experiencing a mNT injury only (WT-1 vs. GD3sKO; $P = 0.45$) and both groups experiencing a combined mNT and pSC-injury (WT-2 vs. GM2sKO; $P = 0.12$). The same was observed for the aged experimental groups (WT-1 vs. GD3sKO, $P = 0.20$; WT-2 vs. GM2sKO, $P = 0.53$).

DISCUSSION

WT mice appear predominantly to express complex gangliosides (including GD1a) in their mNTs and simple gangliosides (including GD3) in their pSCs (Halstead et al., 2005b; Rupp et al., 2012). Therefore,

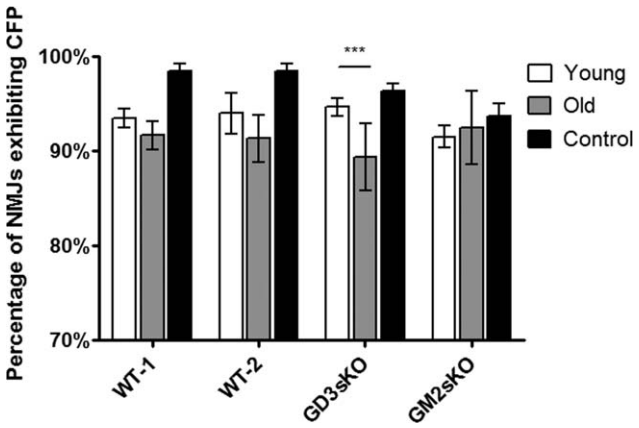

Fig. 4. Extent of mNT regeneration (assessed by CFP overlying the NMJ) following 5 days of regeneration in young and aged WT, GD3sKO, and GM2sKO mice. Due to ganglioside expression and specificity of the anti-ganglioside Abs applied, WT-1 and GD3sKO mice experienced a selective mNT injury, whereas WT-2 and GM2sKO mice experienced a combined mNT and pSC injury. Comparing the young and aged mice undergoing mNT regeneration, a statistically significant difference was only observed for the GD3sKO mice ($P = 0.0009$; Fisher's exact test, $n = 3$). Statistically, no significant differences were observed between the young or aged experimental groups experiencing mNT injury only (WT-1 vs. GD3sKO, young: $P = 0.45$, aged: $P = 0.20$; Fisher's exact test, each $n = 3$) and groups experiencing a combined mNT and pSC injury (WT-2 vs. GM2sKO, young: $P = 0.12$, aged: $P = 0.53$; Fisher's exact test, each $n = 3$). Data for each group is presented as mean and standard error of the mean.

the application of an anti-GD1a Ab, in conjunction with a source of complement, leads to a selective injury of the mNTs, whereas the joint application of Abs binding both GD1a and GD3 in conjunction with a source of complement is associated with a combined mNT and pSC injury. GM2sKO mice express no complex gangliosides, inferring that in these mice the truncated ganglioside biosynthesis in the motor neurone cell body limits the mNT repertoire to simple gangliosides. The application of an anti-GD3 Ab, therefore, leads to an injury of both mNTs and pSCs. In GD3sKO mice, the mNTs are still able to express 0- and a-series gangliosides; thus, the application of an anti-GD1a Ab, in conjunction with a source of complement, results in a selective mNT injury.

When comparing the extent of mNT regeneration between young adult and aged mice of the different strains and injury types, only very subtle differences were observed. This may be due to the fact that the regenerating mNTs only need to extend a very short distance in this specific experimental paradigm. Similar investigations involving the application of black widow spider venom (α-latrotoxin) which induces a mNT injury very closely resembling anti-ganglioside Ab- and complement-mediated injury in its mechanism, morphology, and time-frame of regeneration, also indicate only very subtle differences in the rate of mNT regeneration when comparing 5–7- and 27–30-month-old mice (Robbins et al., 1990). The slightly lower rate of mNT regeneration found in aged WT and GD3sKO mice when compared with their younger counterparts may reflect the observation that mNT sprouting is decreased in aged animals (when compared with young animals), and that the average rate of regeneration also slows with increasing age (Hopkins et al., 1986; Pestronk et al., 1980). This also might serve as a potential explanation for the poorer clinical prognosis of aged GBS-patients when compared with younger GBS-patients (Durand et al., 2006; Rajabally and Uncini, 2012; van Koningsveld et al., 2002, 2007; Walgaard et al., 2011).

Previous investigations in WT mice have shown that the acute anti-ganglioside Ab-mediated injury remains very localized to the area of the NMJ, whilst over the following 24 h injury extends proximally up to a maximum of 200 μm (Rupp et al., 2012). Considering a one-day time-point was not investigated in this study, it is not known whether this proximal extension varies between strains.

When assessing the extent of acute mNT injury (as assessed by the percentage of NMJs exhibiting the normal appearance of unfragmented CFP overlapping the bungarotoxin signal), and bearing the two different types of injuries in mind (mNT or combined mNT and pSC injury), statistically significant differences were observed between the different strains. The ensuing extent of mNT regeneration was comparable between the different strains experiencing the same type of injury. At the same time, previous and current investigations conducted in young adult and aged WT mice have indicated that the rate of mNT regeneration in this experimental setting is independent of healthy pSCs overlying the NMJ (Rupp et al., 2012). Considering pSCs have been shown to major play a role in supporting reinnervation of the NMJ following traumatic denervation (Son and Thompson, 1995; Son et al., 1996) this finding is rather unexpected. To account for this, we previously have suggested that either the anti-ganglioside Ab- and complement-mediated injury to the pSCs is sublethal or the complement-killed pSCs are very rapidly replaced by immature precursors. In both these situations, a pSC population may be able to support mNT regeneration (Rupp et al., 2012). Similar to observations made following denervation and reinnervation of traumatically denervated NMJs (Love and Thompson, 1998; Reynolds and Woolf, 1992), the (immature) pSCs in the current experimental setting also extend processes beyond the NMJ-boundaries and increased numbers of GFP-positive structures overlying the reinnervated NMJs are found on day 5 (Rupp et al., 2012; see also Fig. 2B).

Considering the relative comparability of the extent of mNT regeneration in the different strains (WT-1 vs. GD3sKO and WT-2 vs. GM2sKO), similar findings were observed in a study investigating the role of

gangliosides and the effect of anti-ganglioside Abs in peripheral nerve regeneration. Here, the amount of regenerating fibres at the sciatic level (i.e., close to the site of injury) was comparable between WT, GM2sKO, and GD3sKO mice (Lehmann et al., 2007). Interestingly, at the tibial level (a point further distal), fewer regenerating myelinated fibres were observed in GM2sKO mice when compared with the other two strains, indicating that complex gangliosides might be necessary for regeneration over longer distances and that subtle differences in regeneration cannot be detected over short distances, such as those covered by the regenerating mNTs in this study. Alternatively, the regeneration of distal nerve and mNT-components may rely less heavily on the presence of complex gangliosides when compared with the mechanisms of long distance axonal regeneration, or can be compensated for by the overexpression of simple gangliosides (Takamiya et al., 1996). Assessment of intermediate timepoints (e.g., 2 or 3 days) after induction of the injury might shed further light on this.

We have previously shown that the presence of CFP following 5 days of regeneration coincides with a restoration of the normal morphology of the NMJ at the ultrastructural level; however, it remains possible that the crude evaluation of the presence/absence of CFP used as a global indicator for mNT restoration in this study might be inadequate to detect subtle differences between the strains and between young adult and aged mice. Considering, however, that young adult WT mice require 5 days to regenerate their mNTs following a single anti-ganglioside Ab-mediated injury (Rupp et al., 2012) and taking the apparent roles of age and complex gangliosides in axonal regeneration described above into account, one would not expect aged or KO mice to regenerate faster than young adult WT mice. Also containment of the CFP within the mNTs indicates that at least the gross structure including the axolemmal integrity has been re-established. Alternatively, exacerbation of the insult by repeated applications of anti-ganglioside Abs and complement, or the possible proximal extension of the lesion following a breakdown of the blood–nerve barrier, might be able to illuminate any differences in the regenerative capacities of young adult versus aged mice or WT versus ganglioside-deficient mice, whereas at the same time being able to mimic the clinical situation in GBS-patients more accurately.

Out of all groups, the aged GD3sKO mice performed least well with respect to mNT regeneration capacity, consistent with previous reports that these mice have impaired peripheral nerve regeneration (Okada et al., 2002). Following peripheral nerve resection, the application of b-series ganglioside GT1b in (WT) rats appears to be more effective in preventing neuronal death and promoting nerve regeneration when compared with other gangliosides (Itoh et al., 2001). Also, the application of GT1b in mice lacking complex gangliosides also markedly increases the number of surviving neurons and promotes nerve regeneration (Kittaka et al., 2008). The overexpression of a-series gangliosides such as GM1, which has been shown to be neurotrophic (Doherty et al., 1985; Duchemin et al., 2002), may be able to compensate for the lack of b- and c-series gangliosides in young GD3sKO mice.

In summary, this study has shown that the rate of mNT regeneration—following an anti-ganglioside Ab- and complement-mediated injury and independent of the presence of healthy pSCs overlying the NMJ—does not differ majorly between young adult or aged mice. Aged mice exhibited only a slight tendency for slower or less complete mNT regeneration. At the same time, mice deficient in b- and c-series gangliosides and mice deficient in all complex gangliosides regenerate equally well as WT mice in the current experimental setting. These findings suggest that either the very discrete and localized events mediating mNT regeneration do not rely heavily on complex gangliosides, or b- and c-series gangliosides or that the lack of either of these might successfully be compensated for by overexpression of the remaining gangliosides in these mice.

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