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Research Article

Role of metallothioneins in peripheral nerve function and regeneration

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Abstract. The physiological role of the metallothionein (MT) family of proteins during peripheral nerve injury and regeneration was examined in *Mt1+2* and *Mt3* knockout (KO) mice. To this end, the right sciatic nerve was crushed, and the regeneration distance was evaluated by the pinch test 2–7 days postlesion (dpl) and electrophysiologically at 14 dpl. The quality of the regeneration was assessed by light microscopy and immunohistochemical methods. The results show that the regeneration distance was greater in the *Mt3* KO than in the *Mt1+2* KO mice, whereas control mice showed intermediate values. Moreover, the number of regenerating axons in the distal tibial nerve was significantly higher in *Mt3* KO mice than in the other two strains at 14 dpl. Immunoreactive profiles to protein gene product 9.5 were present in the epidermis and the sweat glands of the plantar skin of the hindpaw of the *Mt3* KO group. The improved regeneration observed with the *Mt3* KO mice was confirmed by compound nerve action potentials that were recorded from digital nerves at 14 dpl only in this group. We conclude that *Mt3* normally inhibits peripheral nerve regeneration.

Key words. Nerve regeneration; sciatic nerve; metallothioneins; growth inhibitory factor; crush.

Introduction

Metallothioneins (MTs) are low-molecular-weight, heavy-metal-binding proteins, which have been suggested to be physiologically relevant in the central nervous system (CNS) during injury [1–3]. In rodents, the MT family is comprised of four isoforms, MT-I to MT-IV [3]. MT-I+II are widely expressed, including in the CNS, and are regulated coordinately, while MT-III and MT-IV are expressed predominantly in the CNS and squamous epithelia, respectively. MT-III was discovered unexpectedly as a neuronal growth inhibitory factor that was depleted in people with Alzheimer's disease [4], but the latter result is in doubt [5–8]. Nevertheless, MT-III expression in the brain is significantly altered following brain injury [9–17], suggesting that it is important for coping with CNS injuries. Recently, MT-III has again been reported as a growth inhibitory protein for the growth of axons and dentrites in cultures of neurons in the presence of brain extract [18]. A number of studies suggest that the MT-I+II isoforms may also be important in the CNS. Thus, their expression in the CNS is increased by stress and the inflammatory factor endotoxin [19, 20], in neurodegenerative disorders such as Alzheimer's disease, Pick's disease and amyotrophic lateral sclerosis [21–25], or following

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injury caused by the glutamate analogues kainic acid and NMDA [26–29], ischemia [30, 31] and cryolesions [17, 32–34]. Moreover, *Mt1+2* expression is increased in animal models of amyotrophic lateral sclerosis [35] and multiple sclerosis [36] [for review see ref. 3].

The above results suggest that MTs may help neurons and glia cope with the tissue damage elicited by a wide array of factors and diseases, but the specific role(s) of the different CNS MT isoforms still remains to be fully elucidated. The development of mice carrying null mutations in the *Mt1+2* [37] and *Mt3* [38] genes (*Mt1+2* KO and *Mt3* KO, respectively), as well as mice overexpressing them [38, 39], has provided useful information. However, nothing is known regarding their role(s) in the peripheral nervous system (PNS). The aim of the present report was to evaluate the putative physiological importance of MTs in peripheral nerve function and in its regeneration after injury. The results indicate significant but different roles of MT-I+II and MT-III and further highlight the neurological importance of this family of proteins.

Material and methods

Animals

Homozygous *Mt1+2* KO and *Mt3* KO mice raised in the 129/Sv genetic background [37, 38] were used. Control mice were therefore wild-type 129/Sv mice. All mice were kept under constant temperature and had free access to food and water. All experiments were carried out in a humane manner and were approved by the Ethical Committee of the Autonomous University of Barcelona.

Sciatic nerve lesion and functional evaluation of nerve regeneration

Operations were performed under pentobarbital anesthesia (50 mg/kg, intraperitoneally) in three groups of 30 female mice each – control 129/Sv, *Mt1+2* KO and *Mt3* KO mice – all aged 4 months. The right sciatic nerve was exposed and crushed three times, 30 s each time, with fine forceps (Dumont no. 5). The lesion site was kept a constant 43 mm from the tip of the third digit, by lying a measured thread over the anatomical traject of the sciatic nerve. The crush site was labeled by one 10-0 suture stitch through the epineurium of the peroneal branch. The wound was closed with silk sutures (5-0) and disinfected. At 2, 4 and 7 days postlesion (dpl), six to eight mice of each group were anesthetized, and the sciatic-tibial nerve was exposed from the trochanter to the ankle. The regeneration distance of the sensory axons was evaluated with the pinch test. The nerve was successively pinched with fine forceps at 0.5-mm intervals from distal to proximal until a reflex response, consisting of muscle contraction of the trunk, was elicited [40–42]; this point was labeled with one epineurial 10-0 suture. The positive pinch response results from the activation of the growing tips of regenerating axons, which are far more sensitive to mechanical stimulation than more proximal regions of the regenerated nerve [42]. The distance between the two suture-labeled points was measured before and after harvesting the nerve.

The remaining mice were tested at 14 dpl for evidence of reinnervation at the hindpaw [43]. Muscle reinnervation was assessed by stimulating the sciatic nerve through a pair of needle electrodes at the sciatic notch, and recording the compound muscle action potential (CMAP) from gastrocnemius and plantar muscles with microneedle electrodes. The evoked CMAP was amplified and displayed on a digital oscilloscope (Tektronix 420) at settings appropriate to measure the amplitude and the latency time. For sensory nerve conduction tests, the sciatic nerve was also stimulated at the sciatic notch and the compound nerve action potentials (CNAPs) were recorded with small needle electrodes from the tibial nerve at the ankle and from the fourth digital nerves; they were displayed on the oscilloscope to measure the amplitude from onset to peak and the latency to the onset. Functional tests were performed at the same time in the contralateral intact hindlimb as control values for each group. Recovery of pain sensitivity was tested on awake animals by light pricking with a needle at progressively distal points on the plantar aspect of the medial side of the denervated paw. Six areas were tested, from the ankle to the tip of the second digit, under a dissecting microscope. The normal response was vocalization and withdrawal of the paw. On each test, the contralateral paw was tested for comparison. A global score to pinprick (PP) was constructed by addition of the observed response in each of the six areas, subjectively graded from no response (0), reduced or inconsistent responses (1), to normal reaction (2) [43].

Morphological evaluation of nerve degeneration and regeneration

After functional assessment, the mice were perfused transcardially with 4% buffered paraformaldehyde. A segment of the tibial nerve above the ankle was dissected and fixed in 3% glutaraldehyde-3% paraformaldehyde in cacodylate buffer (0.1 M, pH 7.4, 4 h), rinsed in buffer and postfixed in OsO_4 (2%, 2 h, 4 °C), dehydrated through an ascending series of ethanol (70, 90, 96 and 100%) of 15 min each and contrasted with uranyl acetate (2.5%, overnight). The nerve samples were embedded in Agar 100 resin and sectioned transversely using an LKB III 6802 ultramicrotome (Leica). Semithin sections (0.5 μm) were stained with toluidine blue and examined by light microscopy (Olympus BX-40). Images of the whole tibial nerve were acquired at ×10 with an Olympus DP50 camera attached to a PowerMacintosh computer, while sets of images chosen by systematic random sampling of squares representing at least 40% of the nerve cross-sectional area were acquired at ×100. Measurements of the

cross-sectional area of the whole nerve and counts of the number of degenerating and regenerated myelinated fibers were carried out using NIH Image 1.62b software.

Immunohistochemical evaluation of cutaneous innervation

Plantar pads of the hindpaw were removed and processed for immunofluorescent localization of tissue antigens. Specimens were fixed in Zamboni's solution, cryoprotected in 0.2 M Sorenson's buffered solution (pH 7.2), frozen and sectioned at 40 µm thick, then incubated in primary antisera to the neuronal marker protein gene product 9.5 (PGP) (1:800; Ultraclone). After three consecutive washes in PBS-Triton for 1 h, sections were incubated in secondary antibody, donkey anti-rabbit cyanine 3.18-labeled immunoglobulin G (1:200; Jackson Immunoresearch). Samples were dehydrated through alcohols, cleared with methylsalicylate and mounted in dextropropoxiphene (Fluka). Control samples were processed in parallel as described, but without primary antibody. Samples were viewed with an Olympus BX-40 microscope equipped for epifluorescence using appropriate filters and the images were collected with a Confocal Imaging System (Leika TCS-4D).

Statistical comparisons

All results are shown as mean and SE. Comparisons between groups were made by ANOVA followed by Fisher's post hoc test and the differences were considered significant when $p < 0.05$. Regression analyses were performed from the regeneration distance (in mm) at each data collection period and the number of hours postlesion. A linear regression was made to assess the slope of the regeneration rate from the pinch test measurements in each group. The delay (or latency time), expressed in hours, was determined from the intersection of the regression line with the x-axis.

Results

Assessment of axonal regeneration

The results from the pinch test are shown in figure 1 and table 1. Two-way ANOVA with strain and postoperative time as factors gave a significant difference in the advance of regenerating axons between *Mt3* KO and 129/Sv mice ($p = 0.034$) but not between *Mt1+2* KO and 129/Sv mice. The regression lines of the regeneration distance versus time were similar between the three strains, although the slope was slightly higher in the *Mt3* KO mice and lower in the *Mt1+2* KO mice than in the control mice. From these fits, the initial latency of regeneration was calculated to be between 33 and 36 h and the rate of regeneration over the first week after injury was significantly faster in *Mt3* KO mice (3.11 mm/day) than in *Mt1+2* KO mice (2.96 mm/day), with intermediate values for control mice (3.08 mm/day).

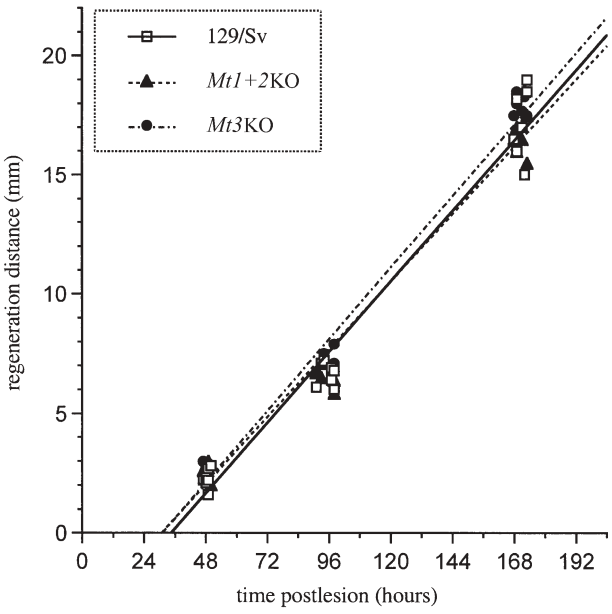

Figure 1. Regeneration distance measured by the pinch test after a crush lesion to the sciatic nerve in the three groups of mice studied.

Table 1. Measurements of the regenerated distance (in mm) and the calculated rate of regeneration (mm/day), assessed by the pinch test, at 2, 4 and 7 dpl in the three groups of mice.

	Distance (mm)			Rate
	2 dpl	4 dpl	7 dpl	(mm/day)
129/Sv	2.28 ± 0.14	6.64 ± 0.16	17.17 ± 0.67	3.08 ± 0.12
Mt1+2 KO	2.53 ± 0.13	6.51 ± 0.17	17.00 ± 0.48	2.96 ± 0.08
Mt3 KO	2.73 ± 0.08	6.99 ± 0.23	17.71 ± 0.16	3.11 ± 0.03

Two-way ANOVA with strain and time post-esion revealed that *Mt3* KO but not *Mt1+2* KO were different from controls ($p < 0.05$).

The Results from the nerve conduction tests performed are shown in table 2. In the intact left sciatic nerve, the amplitude and the latency of the evoked CMAPs and CNAPs did not differ significantly between groups. The electrophysiological tests performed at 14 dpl in the operated limb showed evidence for reinnervation of the gastrocnemius and the interosseus muscles in all animals. The amplitude of the gastrocnemius CMAP was significantly higher in *Mt3* KO than in control mice. There was also a recordable CNAP from the tibial nerve at the ankle in all the mice, with an amplitude of about 10–20 % of normal values. The mean value of the latency was lower in *Mt3* KO than in *Mt1+2* KO. Moreover, CNAPs from the digital nerves were recorded in four out of eight *Mt3* KO mice but in none of the 129/Sv or *Mt1+2* KO mice. The nociceptive-response score was also significantly higher in *Mt3* KO than in *Mt1+2* KO mice.

Table 2. Results of functional tests performed at 14 dpl in the operated and in the contralateral intact hindlimbs.

	129/Sv	*Mt-1+2* KO	*Mt-3* KO
Intact sciatic nerve			
Plantar CMAP			
Latency (ms)	1.78 ± 0.06	1.70 ± 0.08	1.81± 0.11
Amplitude (μV)	6.20 ± 0.50	5.7 ± 0.6	6.5 ± 0.6
Gastrocnemius CMAP			
Latency (ms)	1.08 ± 0.05	0.97 ± 0.08	1.08± 0.07
Amplitude (μV)	47.7 ± 3.1	54.2 ± 5.0	55.2 ± 4.0
Tibial CNAP			
Latency (ms)	0.76 ± 0.04	0.74 ± 0.03	0.69 ± 0.03
Amplitude (μV)	270 ± 24	264 ± 32	352 ± 42
Digital CNAP			
Latency (ms)	1.35 ± 0.04	1.28 ± 0.04	1.29 ± 0.03
Amplitude (μV)	29.8 ± 2.20	30.7 ± 3.5	31.5 ± 1.7
Crushed sciatic nerve			
Plantar CMAP			
Latency (ms)	15.1 ± 1.0	16.2 ± 1.1	15.3 ± 0.40
Amplitude (μV)	0.12 ± 0.03	0.11 ± 0.30	0.14 ± 0.04
Gastrocnemius CMAP			
Latency (ms)	4.5 ± 0.4	4.6 ± 0.2	4.3 ± 0.3
Amplitude (μV)	3.3 ± 0.4	3.8 ± 0.4	5.2 ± 0.9*
Tibial CNAP			
Latency (ms)	2.32 ± 0.59	2.93 ± 0.78	1.91 ± 0.24†
Amplitude (μV)	53 ± 5.3	40.3 ± 9.0	50.1 ± 4.4
Digital CNAP			
Latency (ms)	—	—	5.68 ± 1.0*†
Amplitude (μV)	0	0	2.6 ± 1.6*†
Nociception	2.8 ± 0.4	2.5 ± 0.5	4.0 ± 0.6†

* $p < 0.05$ vs 129/Sv, † $p < 0.05$ vs *Mt1+2* KO.

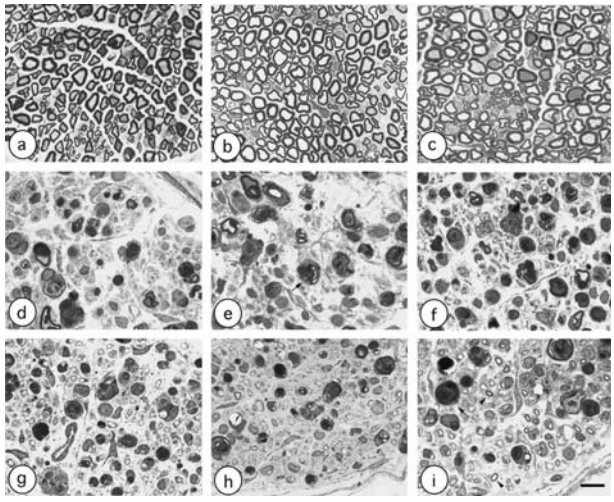

Figure 2. Representative micrographs of transverse sections of the tibial nerve at the ankle in intact nerves (*a–c*) and in nerves at 7 (*d–f*) and 14 (*g–i*) days after sciatic nerve crush in 129/Sv (*a, d, g*), *Mt1+2* KO (*b, e, h*) and *Mt3* KO mice (*c, f, i*). Arrows indicate degenerating myelinated fibers; arrowheads indicate regenerated myelinated fibers. Bar, 10 μm.

Morphological assessment

Nerves from the intact left hindlimb of the three experimental groups showed a similar number of myelinated fibers and cross-sectional area (see figs. 2, 3). The morphology and size distribution of the myelinated fibers did not differ between the groups.

From 2 to 7 dpl, the right tibial nerves showed progressive evidence of degeneration, with a loss of myelinated fibers and an increasing proportion of fibers undergoing Wallerian degeneration. The proportion of myelinated fibers with a normal appearance decreased from 99% at 2 dpl to 46% at 4 dpl and to 8% at 7 dpl. The degeneration process did not show noticeable differences between the three strains of mice evaluated, although at 7 dpl, the proportion of degenerating fibers was higher in *Mt3* KO mice than in those of the other two strains (figs. 2, 3). During the course of degeneration, the shape of the myelinated fibers became irregular, the myelin sheaths were wrinkled and disorganized and the axons diminished in size. The numer of denervated Schwann cells, fibroblasts and macrophages increased. The endoneurial tissue appeared looser than in control nerves, and the cross-sectional area of the nerve increased after lesion.

At 14 dpl, there were still some degenerating myelinated fibers in all the nerves. However, at this time point, there were numerous regenerated fibers, with significantly more in *Mt3* KO mice than in control and *Mt1+2* KO mice (fig. 3). The regenerated fibers were of small diameter, had a thin myelin sheath and were often cut at the Schwann cell nucleus level indicating that they have short internodes.

Reinnervation of the skin

Immunoreactivity to PGP was seen in nerve profiles of the *Mt3* KO strain, throughout the hindpaw pads in a subepidermal nerve plexus that gives rise to small nerve branches that penetrate the epidermis, and in a network that innervates the sweat glands (fig. 4). In contrast, no detectable PGP immunoreactive nerve profiles were evident in *Mt1+2* KO and 129/Sv strains (fig. 4).

Discussion

A crush to a peripheral nerve causes significant damage which induces a set of responses that help limit the injury and initiate functional recovery of the nerve [44–46]. The lesion produced by crushing a peripheral nerve causes complete axotomy but does not disrupt the basal lamina surrounding each nerve fiber or the epineural sheaths [47], and the maintenance of a continuous basal lamina provides proper guidance for regenerating axons to reach their targets. Thus, the crush leads to Wallerian degeneration of the distal nerve stump, and nerve fibres subsequently regrow from the site of injury. For nerve fibers

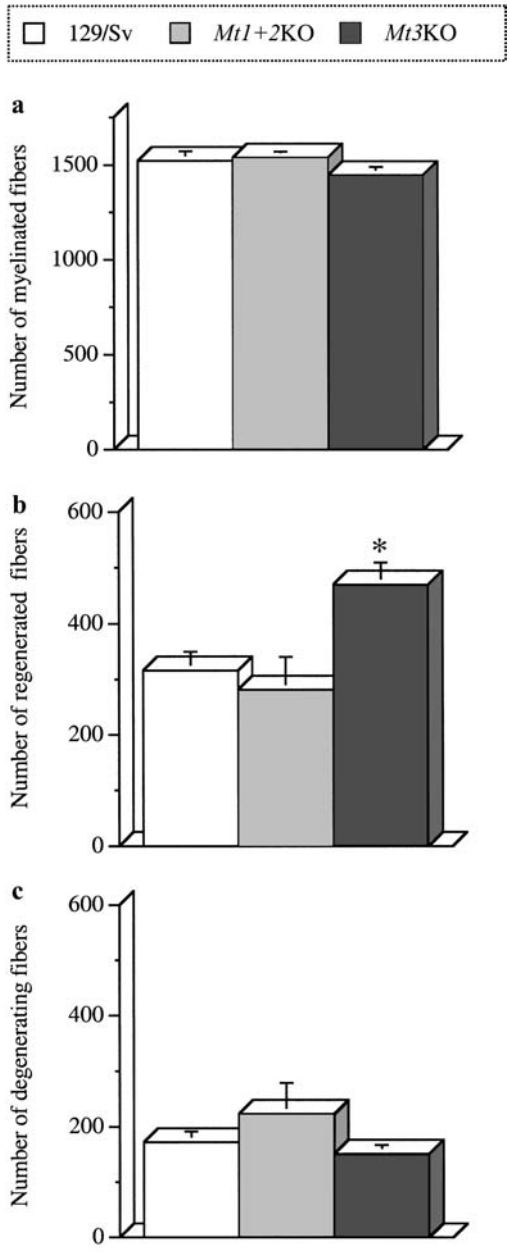

Figure 3. Number of myelinated fibers in the left intact tibial nerve (*a*), number of regenerated (*b*) and degenerating myelinated fibers (*c*) in the right tibial nerve of the three mouse strains at 14 days after sciatic nerve crush. *$p < 0.05$ with respect to 129/Sv and to *Mt1+2* KO mice.

undergoing regeneration, a profound change in the pattern of gene expression occurs in the neurons. In addition, Schwann cells extrude their myelin sheaths, downregulate myelin genes, dedifferentiate and proliferate, and activate a number of genes that are essential for the growth process of nerve fibers [48]. Invading cells such as macrophages also play a role, not only by removing myelin debris and degenerated axons, but also by stimulating the regenerative process.

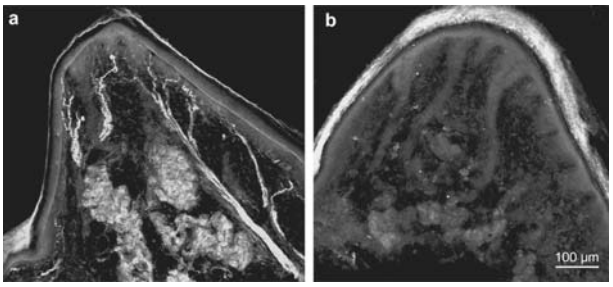

Figure 4. Confocal micrographs of PGP immunofluorescence in samples of *Mt3* KO (*a*) and 129/Sv (*b*) mice. Immunoreactive nerve profiles in *Mt3* KO foot pad (*a*) are seen around the sweat glands, in subepidermal nerve plexus and in epidermal layers, whereas in 129/Sv foot pads (*b*) there were no detectable immunoreactive nerve profiles. Bar, 100 µm.

Characterization of the factors involved in peripheral nerve regeneration is of major importance, and here we examined the importance of the MT family of proteins. Our results show that the rate of axonal regeneration was around 3 mm/day, in accordance with previous results using responses to the pinch test [40, 49–51], axonal transport labeling [52, 53] and immunohistochemical labeling [54] to measure axonal growth in the sciatic nerve of rodents. The present results suggest that, overall, the *Mt3* KO mice show a higher level of reinnervation than the *Mt1+2* KO mice and their proper genetic control mice (129/Sv). Thus, the pinch test showed a significantly faster regeneration in the *Mt3* KO mice when considering all the animals studied. We acknowledge that if results are compared at each time interval, they suggest a greater effect of MT-III deficiency at earlier times postlesion, but this is likely related more to the fact that different animals were studied at each time period (and thus to interindividual variability) than to a time-dependent effect of MT-III. To further support this point, we need to keep in mind that the pinch test evaluates the front of leading regenerating axons, and thus a positive response may be obtained with only a few axons grown to the pinched point. Altogether, these methodological problems indicate the need to evaluate all animals together rather than analyzing small groups, despite the obvious importance of identifying time-dependent effects, which was not the main aim of this study.

Further support for faster regeneration following peripheral nerve injury in *Mt3* KO mice came from the electrophysiological and morphological results. The evaluations performed in the intact left sciatic nerve did not show any noticeable difference between the three strains of mice studied. In addition, the general motor and sensory behaviors of the two different *Mt* KO mice groups did not differ from those observed in their 129/Sv genetic background, further suggesting that there are no phenotypic changes in the structure and function of the PNS induced by MT deficiencies. This also seems to be the case in the

CNS [38, 39]. In response to the crush lesion, a different pattern emerged, with clear signs of a significant role for MT-III but not MT-I+II for the regenerative process of the damaged nerves. Thus, by 14 dpl, tibial CNAP latency was shorter in the *Mt3* KO mice compared to control and *Mt1+2* KO mice, and digital CNAPs were recorded only in *Mt3* KO mice. These results are consistent with those obtained in the pinch test. In contrast to tibial and digital CNAP latencies, gastrocnemius and plantar CMAP latencies were not different in the *Mt3* KO mice compared to control mice. This might be explained by the higher sensitivity of measurements of latency (or nerve conduction velocity) in sensory nerves, in which the recording is made on the nerve, than in motor nerves, in which the recording is made in the innervated muscle and therefore the latency includes the synaptic delay time. A somewhat opposite pattern was seen for nerve and muscle action potential amplitudes. Thus, gastrocnemius CMAP but not tibial CNAP amplitude was increased in the *Mt3* KO mice. The putative mechanisms underlying such effects are unknown and deserve further attention. The electrophysiological data suggested faster nerve regeneration in the *Mt3* KO mice, and this was substantiated by the morphological findings of a higher number of regenerated myelinated fibers in the tibial nerve at 14 dpl and by the reinnervation of epidermis and sweat glands of the pads observed only in *Mt3* KO mice. Thus, this study demonstrates a role for MT-III in inhibiting peripheral nerve regeneration in vivo.

The putative mechanisms underlying the inhibitory effect of MT-III on peripheral nerve regeneration are likely to be complex and at this point are difficult to discuss. For example MT-III may play an inhibitory role in neuronal survival. Indeed, MT-III was originally called growth inhibitory factor (GIF) because it inhibits survival of cultured neurons in the presence of human brain extracts [4]. The inhibitory activity is a property of the β domain of MT-III and is not observed with other MT isoforms [5, 55, 56]. In contrast to this bioassay, a number of in vitro results support a neuroprotective role MT-III. Thus, we have shown that MT-III decreases glutamate-induced neurotoxicity [57], while other laboratories have also demonstrated a neuroprotective role of MT-III following injuries caused by -amyloid 25–35 peptide [58], by S-nitroso-thiols and H_2O_2 [59] and by high oxygen conditions [60]. Noteworthy is that MT-III has a neuroprotective role in the absence of human brain extracts in the same bioassay originally described by Uchida et al. [4, 5, 18, 56]. Thus, in terms of neuronal survival, the inhibitory role of MT-III depends on its interaction with an unknown factor from human or rat brain extracts. Because MT-III is a cytoplasmic protein that is not known to be secreted, the relevance of these bioassays for the normal physiology of the brain remains to be established, and at present we have to rely on results produced in *Mt3* KO mice. These results indicate a neuroprotective role described for the kainate-induced seizures model [38], and also from results obtained recently with transgenic G93A SOD1 mice crossed with either *Mt1+2*-null or *Mt3*-null mice [61]. The deficiency of MT-I+II or MT-III potentiated motor neuron impairment in the G93A SOD1 mice, but the mechanisms underlying such effects appear to be different. Thus, MT-I+II deficiency causes motor neuron impairment that is not matched by loss of cell number, while MT-III deficiency potentiates motor neuron death. The mechanisms underlying such neuroprotective roles are unknown, but might be related to free radical scavenging or disturbance of zinc homeostasis [38, 61]. Thus, evidence is mounting that MT-III, if anything, increases neuronal survival in vivo. The relevance for the present study, however, is unclear, since the peripheral axotomy induced by nerve crush at the site of injury induces only negligible levels of neuronal death [46, 48]. Rather than inhibiting neuronal survival, MT-III could be inhibiting neuronal sprouting following injury, as recently suggested by in vitro experiments [18]. Our results are consistent with that study; however, those effects were caused by added, extracellular MT-III, while our results were being mediated presumably by intracellular MT-III. Whether exogenous MT-III can be incorporated into neurons and other cells or, instead, can be secreted, remains to be established. The physiological function of intracellular MT-III in the present setting is unknown, but it is clearly different from those of MT-I+II.

In summary, the present study demonstrates for the first time an inhibitory role of MT-III in the PNS following injury. This is a specific effect, not shared by MT-I+II, and unlikely to be due to effects on neuronal survival which nevertheless may play a role in other physiological situations.

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