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Research Article

Metallothionein expression by NG2 glial cells following CNS injury

R. S. Chung*, S. J. Fung, Y. K. Leung, A. K. Walker, G. H. McCormack, M. I. Chuah, J. C. Vickers and A. K. West

NeuroRepair Group, Menzies Research Institute, University of Tasmania, Private Bag 58, Hobart, Tasmania 7001 (Australia), Fax: +61 3 62262703, e-mail: rschung@utas.edu.au

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Abstract. Metallothionein (MT) expression is rapidly up-regulated following CNS injury, and there is a strong correlation between the presence or absence of MT and improved or impaired (respectively) recovery from such trauma. We now report that a distinct subset of NG2-positive, GFAP-negative glial cells bordering the injury tract express MT following focal injury to the adult rat neocortex. To confirm the ability of these NG2 glial cells to express MT, we have isolated and cultured them and identified that they can express MT following stimulation with zinc. To investigate the functional importance of MT expression by NG2 glial cells, we plated cortical neurons onto these cells and found that expression of MT enhanced the permissivity of NG2 glial cells to neurite outgrowth. Our data suggest that expression of MT by NG2 glial cells may contribute to the overall permissiveness of these cells to axon regeneration.

Keywords. Traumatic brain injury, reactive glia, axon regeneration, inhibitory molecules.

Introduction

Metallothionein (MT) is a small zinc-binding protein found in all organs of the body. In the post-natal central nervous system (CNS), MT is mainly expressed in astrocytes, although there are some reports of very low levels of neuronal expression (reviewed in [1, 2]). It is considered an intracellular protein, lacking secretion signal sequences, and its endogenous physiological role is believed to be associated with zinc metabolism or protection against free radical damage (reviewed in [3]). Within humans, there are two main classes of MT found within the CNS: MT-I/MT-II (MT-I/-II, the major focus of this study) and MT-III [1].

Numerous studies have established that MT-I/-II are major neuroprotective and neuroregenerative proteins within the injured CNS. In this regard, transgenic studies have been particularly insightful. For instance, MT-I- and MT-II-knockout mice are susceptible to physical, chemical and ischemic brain injury [4–6], while mice that over express MT-I/-II isoforms in the brain are comparatively more resistant to injury [7]. The mechanism by which MT-I/-II is protective within the injured brain is unclear. Possibilities include regulation of zinc homeostasis (MT binds seven zinc ions per protein molecule) or its ability to scavenge free radicals, both intracellular functions for these proteins. An intriguing possibility that has recently arisen from the work of Penkowa and Hidalgo is that MT-I/-II is able to reduce inflammation associated with CNS injury, leading to enhanced neural recovery [8, 9]. In particular, they have demonstrated that intraperitoneal administration of MT-I/-II reduces inflammatory responses to cortical cryolesion and experimental autoimmune encephalomyelitis, an ani-

* Corresponding author.

mal model of multiple sclerosis. These studies imply that MT-I/-II is acting extracellularly upon immune system cells, although there is no direct evidence yet to support this. Interestingly, we have recently discovered that exogenous human MT-IIA (the major human MT-I/-II isoform) has a previously unsuspected ability to enhance neuronal recovery following injury by directly acting upon neurons [10]. These data suggest that an important component of MT function within the injured brain may involve an extracellular localisation (in addition to any intracellular actions) and that extracellular MT-I/-II might promote neuronal recovery by at least two distinct mechanisms, by decreasing the inflammatory response associated with injury and by directly promoting neuronal recovery. Taken together, these studies suggest that MT-I/-II plays a critical role in the cellular response to brain injury; indeed, it has been shown that mice unable to express MT-I/-II in response to injury have a dramatically impaired recovery from CNS trauma [4]. To explore this further, we have investigated MT-I/-II expression in an experimental model of mild traumatic brain injury. We observed that in addition to astrocytic expression of MT-I/-II following CNS injury, other cells also express considerable quantities of MT-I/-II. We identified some of these cells as NG2-expressing glia and investigated the functional consequences of MT-I/-II expression by these cells.

Materials and methods

Focal injury to the adult rat neocortex. Injuries were made to the Par1 region of the adult Hooded-Wistar rat neocortex as reported previously [10]. Briefly, a small hole was drilled into the skull, and a 25-gauge Hamilton syringe inserted to a depth of 1.5 mm into the Par1 region of the somatosensory cortex to produce the focal injury. At the appropriate time, rats were re-anaesthetised and transcardially perfused with 100 ml PBS followed by 100 ml 4 % paraformaldehyde at a flow rate of 8 ml/min. Brains were removed and post-fixed overnight in 4 % paraformaldehyde at 4°C. They were then embedded in 5 % agar (in 0.1 % PBS) and sectioned by vibratome (Leica VT1000E) at a thickness of 50 μm. Sections were then used immediately for fluorescent immunohistochemistry or were stored in PBS (containing 0.01 % sodium azide) at 4°C. To label astrocytes, rabbit anti-GFAP (1:500; Chemicon) or rabbit anti-S100 (1:500; Chemicon) antibody was used. Activated microglia were visualised using Isolectin-B4 conjugated to Alexa-Fluor-594 (1:1000; Molecular Probes). MT was detected using a mouse anti-MT-I/-II antibody (1:500; Dako). Primary antibodies were visualised using appropriate secondary antibodies conjugated to Alexa-Fluor-488 or -594.

Primary rat NG2 glial cell cultures. The cerebral cortices were dissected from postnatal day 1 rats and transferred to HBSS medium. An equal volume of 0.75 % trypsin collagenase (~2 ml) was added and the cell suspension incubated at 37°C for 25 min. Media was replaced with 2 ml DMEM + 10 % serum (DMEM-10S) and the tissue triturated. The cell suspension was then filtered through a 0.6-μm gauze filter to remove any undigested tissue, and the solution was made up to 10 ml with DMEM-10S and centrifuged for 10 min at 500 × g at 4°C. The supernatant was removed and 1 ml fresh DMEM-10S added, followed by gentle trituration. This cell suspension was added to a poly-L-lysine-coated (1:25 dilution) 75-cm^2 flask containing 9 ml pre-warmed DMEM-10S. The cells were incubated for 24 h in 5 % CO_2 at 37°C before the media was replaced. Media was then replaced every 48 h.

Once the cells had become confluent (approximately 8–10 days), the flask was shaken at 250 rpm, 37°C for 30 min with the lid firmly closed. The media was then changed (removes microglia from the culture), fresh media added and the cells shaken for 24 h with the lid firmly closed. The media, which contained primarily NG2 glial cells, was collected and the cells plated onto glass coverslips coated with 0.01 % poly-L-lysine at a density of 1×10^5 cells/well. Cells were maintained in Neurobasal™ media supplemented with 10 ng/ml PDGF. Only cultures with NG2 glial cells comprising >90 % of the culture were used for experiments. We observed almost no microglia (using immunostaining with isolectin-B4) in our cultures (results not shown) due to their separation and removal during the shaking procedure.

In some experiments zinc was applied to NG2 cells to induce MT-I/-II expression. In preliminary trials, we investigated MT-I/-II induction following treatment with 1, 10 and 100 μM (we routinely use similar levels of zinc to induce MT-I/-II expression in astrocytes) and noted clear up-regulation with the 100 μM treatment. This zinc concentration was used for all experiments in this study.

Primary rat cortical neuron cell cultures. Cortical neuron cultures were prepared as reported previously [10], and cells were plated onto glass coverslips (132 mm^2) pre-coated overnight with 0.01 % poly-L-lysine at a cell density of 5×10^4 cells/well. Cultures were maintained at 37°C in humidified air containing 5 % CO_2. Neurons were initially plated into a culture medium consisting of Neurobasal™ medium supplemented with 10 % foetal bovine serum, 0.1 % (f/c) B-

27 supplement, 0.1 mM (f/c) L-glutamine and 200 U/ml gentamicin. At 24 h post-plating, the media was replaced with similar medium not containing foetal bovine serum.

Transfection of NG2 glial cells. In some experiments NG2 glial cells were shaken from mixed glial cultures (as described above) and 1×10^6 cells transfected with 3 μg of a plasmid expressing either human MT-IIA (the predominantly expressed human MT-I/-II isoform) conjugated to enhanced GFP (EGFP) or EGFP alone (as a control). In both plasmids, the target gene is under the control of the CMV promotor (the pEGFP-N1 plasmid from Clontech). Transfections were performed by electroporation using a rat hippocampal neuron protocol and the Nucleofector™ (Amaxa). MT-EGFP expression in transfected NG2 glial cells was observed throughout the cell body (results not shown).

Quantitative analysis of neurite outgrowth. At the appropriate time point, cells were fixed with 4% paraformaldehyde and immunolabelled for the neuron-specific cytoskeletal protein βIII-tubulin (detected using an AlexaFluor-488 secondary antibody). Digital images were captured on a Leica DMIRB microscope using a Magnafire CCD camera. The images were imported into HCA-Vision (CSIRO Australia), and the following parameters used for the program to automatically identify and measure neurites: neuron body channel = 1, size of prefilter = 6, top hat width = 99, minimum area of neuron body = 225, minimum radius of neuron body = 7. More than 200 neurons were analysed per treatment for each experiment, and the measurements were automatically imported into an Excel spreadsheet. The average total length of all neurites and the average length of the longest neurite of each cell were calculated and statistical *t*-test analysis performed.

Results

Co-localisation of MT-I/-II and NG2 expression following CNS injury. We have recently reported that MT-I/-II expression is not detectable within the uninjured adult rat neocortex but can be detected at 4 days post-injury (dpi) following needlestick injury, peaking at 7 dpi and decreasing by 14 dpi [11]. While we reported that this expression was primarily within astrocytes bordering the injury tract, we have recently observed that, in addition, a distinct layer of MT-expressing cells is present within the lesion area at 7 dpi, forming a boundary between infiltrating microglia and resident GFAP-positive astrocytes and neurons within uninjured tissue bordering the injury tract (Figs. 1, 2). We found that MT-I/-II immunolabelling did not co-localise with GFAP along this border (Fig. 1), suggesting that this MT-I/-II staining is not in astrocytes (although it is important to note that GFAP immunostaining does not label the entire volume of astrocytes [12]). Similarly, we have used isolectin-B4 as a marker of microglia to determine that these MT-expressing cells are not microglia (Fig. 2A). Recent studies have reported that another population of glial cells, those expressing the NG2 proteoglycan, respond to cortical needlestick injury by aligning along the injury tract by 7 dpi [13]. Based upon this information, further studies identified co-localisation of some of this MT-I/-II immunostaining with the proteoglycan NG2 (Fig. 2B). Because of the high levels of immunostaining for both MT-I/-II and NG2, it was difficult to clearly determine a precise cellular co-localisation between NG2 and MT-I/-II, even using confocal microscopy (results not shown). Attempts were made to confirm that these NG2-expressing cells also express MT-I/-II using *in situ* hybridisation techniques that have been successful in mice [14]; however, these techniques were found to be unsuccessful for rat tissue (results not shown).

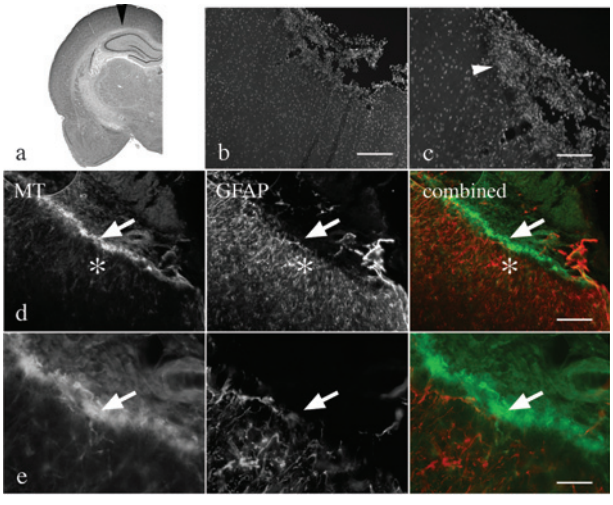

Figure 1. Focal needlestick injuries were performed in the Par1 region of the somatosensory cortex (indicated in the schematic by the black arrowhead, panel *a*). At 7 days post-injury, nuclear staining demonstrated the accumulation of a dense mass of cells bordering (arrowhead) and within the injury site (*b*, higher magnification in panel *c*). MT-I/-II (green) expression (*d*) was up-regulated by astrocytes residing in neural tissue bordering the injury tract (indicated by *). However, an additional band of metallothionein (MT)-rich cells was observed forming a border between uninjured neural tissue and invading microglia (arrow). At higher magnification (*e*), it is clear that these MT-expressing cells (arrow) are not GFAP (red)-positive; indeed, they present a different appearance compared to GFAP immunoreactive astrocytes. The pial surface is to the top right of the field of view. Scale bars = 80 μm (*b*), 40 μm (*c, d*) and 20 μm (*e*).

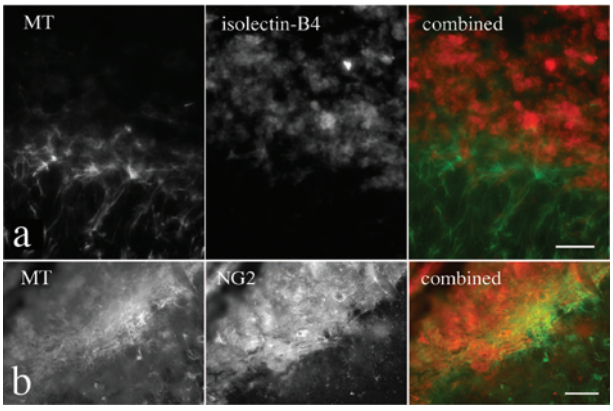

Figure 2. At 7 days post-injury, MT-I/-II (green) expression is not found within activated microglia (isolectin-B4, red) that invade the lesion site (*a*, pial surface at top of picture). The distinct band of metallothionein (MT)-positive (green) immunoreactivity appears to co-localise with NG2 chondroitin proteoglycan (red) immunoreactivity (*b*), although it is difficult to determine cellular co-localisation (pial surface to the top left). Scale bars = 20 μm (*a*), 40 μm (*b*).

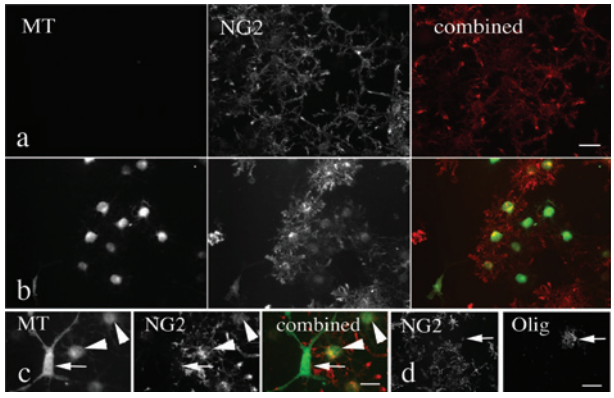

Figure 3. NG2 glial cells were isolated from the postnatal day 2 cerebral cortex and maintained in culture for 3 days. At this time they do not express metallothionein (MT) (*a*). However, treatment with 100 μM zinc for 24 h results in dramatic up-regulation of MT expression (*b*). MT expression was observed within the cell body and processes of NG2 glial cells (arrowheads) as well as astrocytes (arrow, *c*). Mature oligodendrocytes labelled with the marker Olig (arrow) were not NG2 immunoreactive (*d*). Scale bars = 10 μm (*a, b*) and 7.5 μm (*c, d*).

Expression of MT-I/-II can be induced in cultured NG2 glial cells. To determine whether NG2 glial cells can express MT-I/-II, we isolated and cultured these glial cells from the postnatal day 2 cerebral cortex, as per previous reports [15, 16]. Under these tissue culture conditions, we were able to maintain the cells in an NG2-expressing, undifferentiated state (they do not express markers of mature oligodendrocytes, astrocytes or neurons). All NG2-expressing cells exhibited a similar morphology, a distinctly round cell body with numerous processes extended in a star-like pattern (Fig. 3A). We did not observe any co-localisation between NG2 and GFAP in cultures, such that no NG2-expressing cells were found to express GFAP (results not shown). Under basal culture conditions, the NG2 glial cells did not express MT-I/-II (Fig. 3A). However, addition of zinc, a known inducer of MT-I/-II expression in astrocytes, resulted in dramatic up-regulation of MT-I/-II expression within these NG2 glial cells, primarily within the cell body but also to a lesser degree in the processes (Fig. 3B). All NG2-expressing glial cells were observed to express substantial levels of MT-I/-II. It is important to note that the few contaminating GFAP-positive, NG2-negative astrocytes (1–2 %) present in these cultures also up-regulated expression of MT-I/-II (Fig. 3C). The very few mature oligodendrocytes (<1 %) present in these cultures were NG2-negative, were morphologically very different from NG2 glial cells (Fig. 3D) and did not express MT-I/-II following zinc treatment (results not shown).

Expression of MT-I/-II by NG2 glial cells makes these cells more permissive to neuronal outgrowth. What is the functional role of MT-I/-II expression by NG2 glial cells? Interestingly, it is well established that the NG2 molecule itself is highly inhibitory to axonal outgrowth [17, 18]; however, NG2 glial cells (which express NG2 on their cell surface) actually support neurite outgrowth, even in the presence of elevated levels of NG2 [15]. One possible hypothesis to explain this is that NG2 glial cells are capable of expressing growth stimulatory molecules and that changes in the expression of these growth-permissive molecules following CNS injury may alter the growth-inhibitory cellular substrate presented by NG2 glial cells for neurite outgrowth. Since we and others have demonstrated that MT-I/-II is a powerful promoter of axonal regeneration in vitro [10, 19], we investigated whether expression of MT-I/-II by NG2 glial cells alters their permissivity to neurite outgrowth.

NG2 glial cells were obtained from the cerebral cortex of postnatal day 2 rats and were maintained for 2 days as a cellular monolayer, followed by the seeding of embryonic cortical neurons on top of them. In this assay we noted that cortical neurons were able to attach and extend processes upon the NG2 glial layer over 24 h (Fig. 4A, B). Pre-induction of MT-I/-II expression 24 h prior to seeding of neurons resulted in enhanced outgrowth of neuritic processes from seeded cortical neurons (Fig. 4C, D). To quantitate the amount of neurite outgrowth from cortical neurons, cultures were immunolabelled for the neuronal cytoskeletal marker βIII-tubulin and images analysed using HCA-Vision software (CSIRO). By setting appropriate parameters, HCA-Vision was able to identify individual neurons and trace their processes (Fig. 4E, F), allowing automated calculation of neurite length.

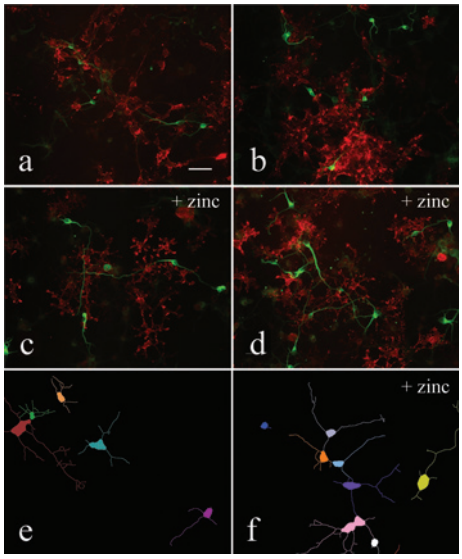

Figure 4. NG2 glial cells (NG2, red) were plated as a feeder layer for embryonic cortical neurons (type III βtubulin, green). After 24 h, neurons had attached to and extended processes along the NG2 cell layer (*a, b*). Pre-treatment of the NG2 glial cells for 24 h with zinc [to induce metallothionein (MT) expression] prior to seeding of cortical neurons resulted in increased neuritic outgrowth (*c, d*). By setting appropriate parameters, HCA-Vision was able to identify individual neurons (in different colours) and trace their processes (*e, f*) for subsequent analysis. Scale bar = 20 μm (*a–d*).

The average total length of all neurites projecting from neurons was significantly increased (by approximately 30 %) when plated upon zinc pre-treated NG2 glial cells (Fig. 5A; $p<0.01$, *t*-test), with the average length of the longest neurite also significantly longer (Fig. 5A; $p=0.015$, *t*-test).

To account for the possibility that the zinc pre-treatment had induced other molecular changes within the NG2 glial cells that contributed to the enhanced permissivity of these cells to neurite outgrowth, we transfected NG2 glial cells with either an MT-EGFP fusion protein-expressing plasmid or an EGFP-expressing plasmid. Cortical neurons were plated onto a feeder layer of these transfected NG2 glial cells (2 days after transfection), and neurite outgrowth of only those neurons growing on top of successfully transfected NG2 glial cells was assessed 24 h later. Neurons plated upon the MT-EGFP-expressing NG2 glial cells projected neurites with significantly greater total length compared to those on EGFP-expressing cells (Fig. 5B; $p<0.01$, *t*-test). Similarly, the average length of the longest neurite was significantly increased (by more than 32 %) when neurons were plated onto MT-EGFP-expressing NG2 glial cells (Fig. 5B; $p<0.01$).

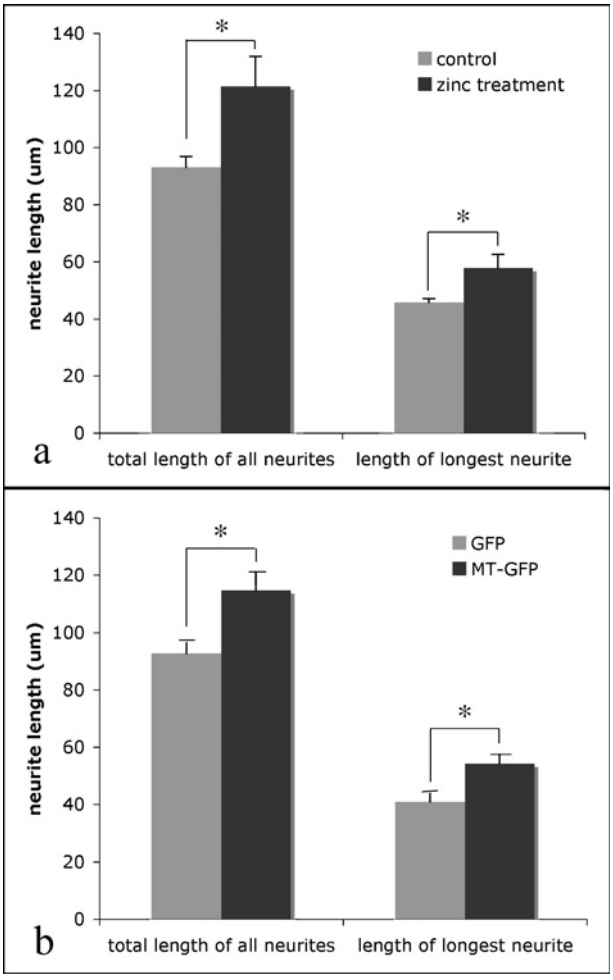

Figure 5. Cortical neurons were plated onto either zinc pre-treated or saline pre-treated NG2 glial cells, and neurite outgrowth was assessed 24 h later. Both the total length of all neurites per neuron and the average length of the longest neurite were significantly greater on zinc pre-treated NG2 glial cells (*a*). In further experiments, cortical neurons were plated onto either MT-EGFP- or EGFP-expressing NG2 glial cells and neurite outgrowth measured 24 h later. Neurite outgrowth was significantly increased on the MT-EGFP-expressing NG2 glial cells (*b*). Values are represented as mean and standard error from three different experiments.

Discussion

We report that NG2-expressing glial cells up-regulate the expression of MT-I/-II following CNS injury and that in culture this facilitates enhanced axonal outgrowth upon the NG2 glial cells. This suggests that MT-I/-II expression by NG2 glial cells may have an important role in the ability of these cells to support axonal regeneration following injury.

Expression of MT-I/-II by NG2 glial cells following traumatic brain injury. It is well established that MT-I/-II expression is rapidly up-regulated following stress to the brain. For example, microarray [20] and serial

analysis of gene expression [21] studies of the injured brain implicate MT-I/-II as major neuroprotective proteins whose expression is up-regulated within the injured CNS. This expression is primarily within reactive astrocytes [11] and, in some situations, microglia (for a recent review see [2]). However, we now report that NG2 glial cells, which do not express markers of other glial cell types such as astrocytes or microglia, form a distinct population of MT-I/-II-expressing cells within the injured brain. The responses of NG2 cells to traumatic brain injury have only recently been reported [13], which might partly explain why MT-I/-II expression by these cells has not been reported previously. In our study, MT-I/-II-expressing NG2 glial cells formed a small but distinct layer separating reactive astrocytes and microglia at the border of the injury tract. They also exhibited a much higher level of MT-I/-II expression than the other surrounding glial cells. Given that we have reported previously that MT-I/-II is a powerful promoter of axonal regeneration [10], one possible hypothesis is that these NG2 glial cells are contributing to the facilitation of axonal regeneration following injury, which might be mediated by the expression of MT-I/-II. This would be in accordance with studies demonstrating that regenerating axons can grow through regions of densely populated NG2 glial cells [22], and indeed we have demonstrated previously that by 21 days post-injury in this model, the injury site is filled with regenerating axons [23]. Interestingly, transgenic mice that cannot express MT-I/-II have impaired regenerative ability [4], which would support our observations, although future studies involving cell-specific targeting/blocking of MT-I/-II expression are required to discern this.

It is very important to address the issue of why NG2 cells have not previously been identified as expressors of MT-I/-II. Indeed, comprehensive work from the labs of Penkowa and Hidalgo has extensively investigated the expression of MT-I/-II following a variety of stresses to the CNS (for a recent review see [24]). In these studies, MT-I/-II expression was observed primarily in GFAP-positive astrocytes as well as microglia. One possible reason for this might be that the model of injury that we have used in this investigation involves a relatively mild physical trauma to the cortex, while the aforementioned studies involved relatively more severe forms of injury. It is possible that in this regard, the response of NG2 glial cells is different relative to the severity of the CNS trauma. Another possibility concerns the recent observation that NG2 glial cells give rise to astrocytes following cortical needlestick injury in the adult rat brain [13], the same model used in this study. It is possible that in previous studies, the time points examined have been after the differentiation of NG2 glial cells into astrocytes has occurred. Furthermore, since NG2 glial cells have only recently been identified as a distinct population of glial cells in the CNS, it is possible that the predominant GFAP-expressing cells identified as producing MT-I/-II after injury may also express NG2 (representing cells in a stage of differentiation between NG2 glial and astroglial).

MT-I/-II expression enhances axonal outgrowth upon NG2 glial cells. To determine the functional significance of MT-I/-II expression by NG2 glial cells, we plated cortical neurons onto NG2 glial cell monolayers. As reported previously [15], NG2 glial cells supported neuronal attachment and outgrowth despite their expression of the inhibitory NG2 molecule on the cell surface. However, NG2 glial cells pre-induced to express MT-I/-II or genetically modified to express MT-IIA provided a much more permissive substrate to neurite outgrowth. Since we and others have reported previously that extracellular MT-I/-II is a powerful promoter of neurite outgrowth in culture [10, 19], one plausible hypothesis is that NG2 glial cells are secreting MT-I/-II, which then acts upon the seeded neurons to promote outgrowth. An alternative possibility is that the nuclear localised MT-I/-II is influencing the gene expression profile of the NG2 glial cells. In this regard, MT-I/-II can act as a regulator of gene expression by controlling the availability of zinc to zinc-dependent transcription factors within the nucleus (see [2] for a review).

The role of NG2 glial cells in axon regeneration. The role of the NG2 proteoglycan in axonal regeneration is still unclear. While it is clear that the NG2 molecule itself is strongly inhibitory to axon regeneration [17, 18, 25], in the context of NG2-expressing cells, this is not always the case; in fact, regenerating axons have been observed passing through regions densely populated with NG2-expressing cells within the injured spinal cord [22], and there is no difference between wild-type and NG2-null mutant mice in the extent of axonal regeneration after spinal cord injury [26]. The ability to culture relatively pure populations of NG2 glial cells has yielded interesting results, with studies finding that these cells can either inhibit axonal growth [27, 28] or, in some cases, support axonal growth, even in the presence of elevated levels of NG2 [15]. One possible hypothesis to explain these observations is that the NG2-expressing glial cells express other molecules that also influence axon regeneration and that it is the balance in expression of these inhibitory and promoting molecules that determines whether an NG2 glial cell will inhibit or promote axon regeneration. In this regard, we present a series of

experiments suggesting that MT-I/-II may be one such promoting molecule and that expression of MT-I/-II by NG2 glial cells has an important role in determining the overall permissiveness of these cells to axon regeneration. We are currently using MT-knockout mice to test this hypothesis by investigating whether these mice have differences in their capacity for axon regeneration compared to wild-type mice.

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