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Microglial inhibitory factor (MIF/TKP) mitigates secondary damage following spinal cord injury

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Abstract

Spinal cord injury (SCI) induces an immune response during which microglia, the resident immunocompetent cells of the central nervous system, become activated and migrate to the site of damage. Depending on their state of activation, microglia secrete neurotoxic or neurotrophic factors that influence the surrounding environment and have a detrimental or restorative effect following SCI, including causing or protecting bystander damage to nearby undamaged tissue. Subsequent infiltration of macrophages contributes to the SCI outcome. We show here that suppressing microglia/macrophage activation using the tripeptide macrophage/microglia inhibitory factor (MIF/TKP) reduced secondary injury around the lesion epicenter in the murine dorsal hemisection model of SCI; it decreased the hypertrophic change of astrocytes and caused an increase in the number of axons present within the lesion epicenter. Moreover, timely inhibition of microglial/macrophage activation prevented demyelination and axonal dieback by modulating oligodendrocyte survival and oligodendrocyte precursor maturation. Microglia/macrophages located within or proximal to the lesion produced neurotoxic factors, such as tumor necrosis factor alpha (TNF- α). These results suggest that microglia/macrophages within the epicenter at early time points post injury are neurotoxic, contributing to demyelination and axonal degeneration and that MIF/TKP could be used in combination with other therapies to promote functional recovery.

Keywords

Hemisection; Spinal cord injury; Mice; Microglia; Oligodendrocytes; MIF/TKP

Introduction

Spinal cord injury (SCI) is a devastating trauma marked by the loss of sensory, motor and autonomic functions due to the degeneration of axons and glia. The initial insult to the central nervous system (CNS) causes necrosis of cells within the lesion epicenter, thereby inducing a cascade of biochemical and cellular events that lead to demyelination, degeneration of spared axons and inflammatory immune responses collectively referred to as secondary damage (Crowe et al., 1997; Liu et al., 1997). The necrosis triggers glial cell (e.g. astrocytes, oligodendrocyte precursor cells, and microglia) activation and recruitment to the site of injury. Glial cell activation and the subsequent gliotic scar formation are thought to

drive the ensuing secondary damage. In this setting, microglia, the resident CNS immune cells, release proinflammatory cytokines, reactive oxygen species and proteases that create bystander damage (Dusart and Schwab, 1994; Popovich et al., 2002). Chemokines produced by microglia, such as CCL2, CCL3, CXCL2/3 and CXCL10 are upregulated in minutes following injury (Rice et al., 2007), recruit and activate peripheral polymorphonuclear leukocytes and monocytes which have been demonstrated to promote neurotoxicity (Carr et al., 1994; Wolpe and Cerami, 1989; Wolpe et al., 1998; Xu et al., 1996). On the other hand, the phagocytic properties of microglia and macrophages ensure myelin and tissue debris removal following the initial insult, which is necessary for proper remyelination of axons and neurite outgrowth (Friede and Brück, 1993). In subsequent phases of the immune reaction, peripheral inflammatory cells such as neutrophils, macrophages and T-lymphocytes enter the lesioned area and release a plethora of cytokines that mediate detrimental effects through the generation of free radicals which promote apoptosis (Hu et al., 1997). While there are some reports that suppression of the initial local inflammatory response following SCI reduces secondary injury around the lesion epicenter and improves functional outcome, other studies have provided evidence that inflammation supports regeneration via growth factors and cytokines released by activated microglia and macrophages. Transplantation of *in vitro* activated microglia or macrophages within the injured spinal cord have been demonstrated to protect tissue, as well as promote axon regeneration and functional recovery via the release of trophic and anti-inflammatory factors (Lazarov-Spiegler et al. 1996; Prewitt et al., 1997; Rabchevsky and Streit, 1997; Rapalino et al., 1998; Schwartz et al., 1999). These restorative effects could be due to the release of trophic factors such as nerve growth factor (NGF), neurotrophin-3 (NT-3), interleukin-1 (IL-1), IL-10, and brain derived growth factor (BDNF), which influence axon regeneration. Reports of heterogeneous subsets of microglia/macrophages render them to be either neurotoxic (M1) or neuroprotective (M2). Subsequent to injury, both M1 and M2 phenotypes are present, however shortly after the initial insult the M1 proinflammatory phenotype predominates, providing evidence that this neurotoxic phenotype may outweigh the neuroprotective phenotype (Guerrero et al., 2012; Kigerl et al., 2009). Thus, therapeutic goals in the context of controlling microglial activation have remained unclear.

Macrophage/microglial inhibitory factor (MIF/TKP, tuftsin 1 fragment 1–3), a tripeptide (Thr-Lys-Pro, TKP) generated during the processing of immunoglobulin G, inhibits activation of cells of monocytic origin. For peripheral macrophages, MIF/TKP decreases cell migration, phagocytosis, chemotaxis and superoxide anion generation (Auriault et al., 1983). MIF/TKP also inhibits microglial function in the CNS. Administration of MIF/TKP during optic nerve trans-section has been reported to reduce axotomy-induced retinal ganglion cell degeneration (Thanos et al., 1993) and to reduce demyelination and T-cell infiltration in the experimental allergic encephalomyelitis (EAE) model of multiple sclerosis, thereby abrogating disease symptoms and reducing lesion volume (Bhasin et al., 2007); MIF/TKP also attenuates edema and decreases neuronal degeneration in intracerebral hemorrhage models (Wang and Tsirka, 2005). Potential beneficial effects of MIF/TKP have not yet been examined in SCI. In this study, mice were treated with MIF/TKP and examined at various time points post SCI to assess consequences of inhibiting microglial/macrophage responses in a severe model of SCI. We demonstrate here that MIF/TKP administration alters the cellular and molecular environment to limit astrocyte hypertrophy, diminish oligodendrocyte cell death, and promote remyelination, leading to decreased secondary damage and axonal dieback.

Materials and methods

Animals

C57/BL6 mice were used in accordance to protocols approved by Stony Brook University IACUC and the Division of Laboratory Animal Research. Two month old C57/BL6 mice were anesthetized using isoflurane and a dorsal laminectomy was performed at T8 thoracic vertebrae. Dorsal hemisections were performed as described by Tan et al. (Nolin et al., 2008; Tan et al., 2006). Briefly, an incision was made in the skin rostrocaudally, which exposed the spinal column. To gain access to the spinal column, muscles and ligaments surrounding the spinal column were torn and cut. Once exposed, the vertebra at the T8 area was filed away with a dental drill to decrease the bone thickness, such that it was possible to break it away from the cord without causing any damage to the cord. The remaining bone was removed with forceps and a micro-scissor. The exposed spinal cord was initially cut in a dextro-sinister direction using a fine (271/2) gauge needle to puncture the dura mater. For consistent hemisections the spinal cords were then cut using a micro-knife connected to a Micro Manipulator (Kopf), which set to an appropriate depth of 0.8 mm. Micro-osmotic pumps (Alzet) that administered PBS or MIF/TKP in PBS (100 µg/g of body weight/day) for 7 days (rate of infusion 0.25 µl/h, 100 µl total volume) or 14 days (rate of infusion 0.5 ml/h, 200 ml total volume) were pre-incubated at 37 °C overnight in saline to ensure that the pumping rate reached steady state prior to placement over the lesioned spinal cord. Following the hemisection the pumps were positioned under the skin and the catheter tubing was sutured under the muscle, so that the contents were distributed over the lesioned area. The skin above the injury site was sutured and the animals were administered buprenorphine and placed on a heating pad until fully recovered from the anesthesia. Bladder expression was performed on the mice at least once daily, and behavioral symptoms (paralysis of the hind legs) were monitored until specific time points post injury when the mice were euthanized and analyzed. Mice that maintained function of one or both legs after the injury were excluded from the experimental analysis, as they were presumed to have undergone incomplete hemisection resulting in axon sparing.

Axon tracing

Retrograde neuronal tracing was performed via injection of cholera toxin Beta-subunit (CTB) following injury. Four days prior to euthanizing, the mice were re-anesthetized, with a constant delivery of isoflurane, and the right sciatic nerve was exposed. Two microliters of a 1.5% solution of CTB (List Biological Laboratories) was injected into the nerve using a Hamilton syringe. The solution was slowly injected over a 1 min period and the syringe was withdrawn over an additional 2 min to prevent reflux. The muscle and skin were then sutured. Spinal cords were isolated, and cryosectioned at 18 µm thickness. Alternating sections were stained against CTB via goat anti-CTB genotoxiod (List Biologicals) and detected with a fluorescently conjugated secondary. The ImageJ software (NIH) was used to measure neurite length. Neurite length was measured and plotted as distance from injury epicenter (0 µm).

Immunohistochemistry

Terminally anesthetized mice were perfused trans-cardially with PBS, followed by 4% paraformaldehyde in PBS (pH 7.4). Spinal cords were isolated, post fixed for 1 h and cryoprotected in 30% sucrose overnight. 18 µm frozen sections were prepared, blocked for 1 h with 5% serum and then incubated with primary antibodies (rabbit anti-IBA-1 1:500, Wako; rabbit anti-GFAP 1:500, Dako; mouse anti-APC (CC1), 1:100, Millipore; rabbit anti-NG2, 1:500 a generous gift from Dr. Joel Levine; goat anti-CTB, 1:5000, List Biologicals; mouse anti-CD3, Dako) overnight at 4 °C. Sections were washed and incubated with a fluorescently conjugated secondary antibody made in goat for 1 h. Sections were mounted

with Fluoromount-G with DAPI. Six sagittal sections every 10 sections (180 μ m) apart were imaged per biological replicate. Two fields in the dorsal white matter (rostral and caudal), two fields in the gray matter (rostral and caudal), and one field in the ventral white matter were taken per section for analysis of astrocytes, microglia, and CSPGs. Two fields rostral and two fields caudal to the lesion were taken in the dorsal white matter and one field of the ventral white matter were imaged per section for sections stained for mature oligodendrocytes. Image J (NIH) was used to quantify the total mean intensity/area for each biological replicate and antibody.

Immunoperoxidase staining

Sections were incubated for 2 min in 3% hydrogen peroxide to block peroxidase activity. Sections were then washed and blocked for 1 h in blocking solution, followed by an overnight incubation of diluted primary antibody at 4 °C. After a second wash series, diluted secondary antibody conjugated to biotin was added to the section for 1 h. Sections were then incubated for 30 min with horse-radish peroxidase–streptavidin ABC reagent (Vector Laboratories). Following wash, sections were incubated with DAB for 5 min or until the chromogen was fully developed. Slides were dehydrated in an ethanol series followed by two 2 min incubations of xylene and mounted in Permount (Fisher).

Immunocytochemistry

Cells were fixed in 4% PFA for 10 min at RT and subsequently washed and blocked for 1 h with 5% serum. Primary antibody was incubated overnight at 4 °C (goat anti-Iba-1, 1:100 AbCam; rabbit anti-NG2 1:500 a generous gift from Dr. Joel Levine; rat anti-MBP 1:100, Serotec; mouse anti CNPase 1:100, Calbiotech). Cells were washed and incubated with secondary antibodies (donkey anti-goat Cy3, donkey anti-rabbit Cy3, donkey anti-rabbit Cy5, donkey anti-rat Cy3, donkey anti-rat FITC, and donkey anti-mouse Cy5 (1:500, Jackson Laboratories)). Cells were washed with PBS and were mounted with Fluoromount-G with DAPI or post fixed with 2:1 ethanol:acetic acid or 2 N HCl for TUNEL and BrdU analysis, respectively.

Measurement of lesion volume

Lesion size was analyzed from digital images of 5 alternate sagittal sections approximately 200 μ m apart (2 sections right of the center of the lesion, 1 section of the approximate center of the lesion and 2 sections left of the center of the lesion) that had been immunofluorescently stained with GFAP, as described above. The lesion was defined as the area devoid of staining bordered by GFAP⁺ immunoreactivity. Tracings of the lesion were performed using the NIH ImageJ program and the area was quantified. The lesion volume was attained by multiplying the lesioned area by the distance between sections.

Tissue collection for immunoblotting and ELISA analysis

To quantify expression of cytokines a separate group of mice was used. Mice were transcardially perfused with PBS. The spinal cords were isolated and immediately frozen at –80 °C.

Immunoblotting

Immunoblots were performed on spinal cord homogenates and total cell lysates. The protein content was quantified by Bradford assay (Bio-Rad). Aliquoted samples were reduced and equivalent concentrations of total protein were separated by SDS-PAGE. The proteins were electroblotted onto low fluorescent polyvinylidene difluoride (PVDF) membranes. Membranes were preincubated with Odyssey blocking buffer (LI-COR Biosciences) in PBS (1:1), and probed with primary antibodies in Odyssey blocking buffer in PBS+ 0.2%

Tween-20 overnight at 4 °C. Membranes were probed with anti-rabbit IR dye 800 (Rockland), anti-mouse Alexa Fluor 680 and anti-rat Alexa Fluor 680 (Molecular Probes Inc.) diluted in PBS-T. The Odyssey Infrared Imaging System (LI-COR Biosciences) was used to detect the proteins of interest for quantification. Quantification was performed with the Odyssey application Software 2.1.

Enzyme linked immuno-absorbent assay (ELISA)

Quantitative determination of TNF- α protein was performed at various days PI. Tissue was homogenized and sonicated. Protein concentrations were determined utilizing the BD OptEIA quantitative sandwich enzyme immunoassay per manufacturer's protocol. Ninety six well plates were coated with diluted Capture Ab (100 μ l per well) and incubated overnight at 4 °C. Then plates were blocked for 1 h at RT with Assay Diluent (200 μ l per well). Standard or sample (100 μ l per well) was added and incubated for 2 h at RT. A working detector (100 μ l per well) was added and incubated for 1 h at RT. Substrate solution (100 μ l per well) was then added and incubated for 30 min at RT in the dark followed by 50 μ l of Stop Solution. Absorbencies of each well were read at a 450 nm light wavelength with a microplate reader.

Primary microglial cell cultures

Cerebral cortices from postnatal day 1 C57BL/6J mice were dissected, digested with trypsin (0.25% in HBSS) for 10 min at 37 °C, and mechanically dissociated by trituration. Cells were plated in DMEM medium, 10% FBS, 1% sodium pyruvate and gentamycin on poly-D-lysine coated dishes. After 10 days, microglial cells were dislodged by the addition of 12 mM lidocaine. Isolated microglia were seeded on poly-D-lysine coated plates.

Primary oligodendrocyte cultures

Cerebral cortices from postnatal day 1 C57BL/6J mice were dissected, digested in HEPES/MEM, 30 units/ml papain, 0.24 mg/ml L-cysteine, and 40 μ g/ml DNase for 60 min at 37 °C, and mechanically dissociated. Cells were plated on PDL coated flasks in DMEM 10% FBS, penicillin, streptomycin and gentamicin. Every 3 days media was changed, at day 10 microglia were shaken off for 1 h at 200 rpm and fresh media was added to culture. To isolate OPCs the flasks were then shaken for 24 h at 350 rpm. To remove any remaining microglia media from the flask was transferred to a 10 cm bacteriologic plastic dish for 30 min. Media was then pipetted several times to remove any loosely attached OPCs. OPCs were then resuspended in SATO (DMEM, 5000 U/ml penicillin/streptomycin, 4 mM L-glutamine (Sigma), 16 μ g/ml putrescin (Sigma), 400 μ g/ml L-thyroxine (Sigma), 400 μ g/ml Tri-iodothyroxine (Sigma), 6.2 ng/ml progesterone (Sigma), 5 ng/ml sodium selenite (Sigma), 100 μ g/ml BSA fraction V (Sigma), 5 μ g/ml insulin (Sigma), and 50 μ g/ml holo-transferrin (Sigma)).

Microglia and oligodendrocyte coculture

Eight well chamber slides were coated with PDL overnight at 37 °C. Chambers were washed and OPCs were plated (20,000 cells/chamber) either in SATO with 0.5% FBS or in SATO with 10 ng/ml PDGF (PeproTech) and 10 ng/ml FGF (PeproTech) to facilitate differentiation or maintain the progenitor population, respectively. After 2 days, microglia were added to the chamber slides and allowed to adhere for 24 h. The cells were then either treated with 20 ng/ml LPS, 5 mM MIF/TKP, or a combination of both and at day 4 cells were fixed with 4% PFA.

Quantification of *in vivo* glial cell proliferation

Ki67⁺ double positive cells were counted for each treatment (n=6/group), where five 40× fields were captured per spinal cord. The proliferation index of microglia (Iba-1), astrocytes (GFAP) and OPCs (NG2) was determined by the number of double positive cells expressing Ki67 divided by the total number of cells positive for the particular cell surface marker (double positive cells/total cells) in each field.

In vitro proliferation assay

Cellular proliferation was examined by using the 5-Bromo-2'-deoxy-uridine Labeling and Detection Kit II (Roche) according to the manufacturer's protocol. Briefly, cell cultures were incubated with BrdU (10 μM/L) and cells were probed for BrdU via anti-BrdU antibody provided and stained with a fluorescently conjugated secondary antibody.

Electron microscopy

Mice were deeply anesthetized and transcardially perfused for 1 h with a Karlsson–Schultz fixative (4% formaldehyde, 2.5% glutaraldehyde, 0.5% NaCl, 0.1 M phosphate buffer pH 7.4). Spinal cords were isolated and post fixed over night with perfusate. Areas of interest were blocked and then sectioned on a vibratome at approximately 50 μm in ice cold PBS. Samples were then placed in 2% osmium tetroxide in 0.1 M PBS, pH 7.4, dehydrated in a graded series of ethyl alcohol and embedded with Durcupan resin in between two pieces of ACLAR sheets (EMS, Hatfield, PA). Ultrathin sections of 80 nm were cut with a Reichert–Jung UltracutE ultramicrotome and placed on formvar coated slot copper grids. Sections were then counterstained with uranyl acetate and lead citrate and viewed with a FEI Tecnai12 BioTwinG² electron microscope at the Central Microscopy Imaging Center in Stony Brook University. Digital images were acquired with an AMT XR-60 CCD Digital Camera system.

Statistics

All surgeries and measurements were performed with an observer blinded to the treatment of the mice. Six sections, every 10 sections (180 μm) apart, were imaged per biological replicate. To compare effects within each treatment or time point, one-way ANOVA was used, as presented in the figure legends, followed by Bonferroni's posthoc test. Data are presented as mean±SEM. **p*<0.05, ***p*<0.01, ****p*<0.001.

Results

MIF/TKP inhibits microglial activation following SCI

To ascertain if MIF/TPK is functionally stable for the time course of the *in vivo* study, in which MIF/TKP is administered for 7 to 14 days via an osmotic pump, MIF/TKP was left at 37 °C for 14 days (14d MIF) and then added to primary microglial cultures to test whether it can still inhibit microglial activation. Its efficiency was compared to fresh MIF/TKP (MIF) treatments. Microglia were treated with 50 mM MIF/TKP and/or 20 ng/ml LPS for 24 h and microglial morphology and TNF-α release was assessed. Non-treated microglia and microglia treated with only MIF/TKP possessed 'resting', ramified morphology. When microglia were treated with LPS the cells became amoeboid suggestive of microglial activation. Only 20% of microglia treated with MIF/TKP+LPS were activated. Treatment of microglia with 14d MIF/TKP resulted in activation of ~45% of microglia. TNF-α and IL-10 levels were quantified using ELISA assays in the cell supernatants. TNF-α production was virtually absent in the non-treated and the MIF/TKP only treatment, and although TNF-α was present in the MIF/TKP plus LPS treatment it was not statistically significant when compared to negative controls. While LPS treatment significantly increased TNF-α

production ($p > 0.001$), LPS plus 14d MIF/TKP caused a reduction in TNF- α by approximately 50% when compared to LPS treatment alone. IL-10 levels remained low. These results indicate that after 14 days MIF/TKP still possessed an inhibitory effect (Fig. 1). The pumps were sutured under the muscle so that the contents diffused over the lesioned area (Figs. 1E and F).

The dorsal hemisection model of SCI creates a severe insult that induces a dramatic hypertrophic glial response (Liu et al., 1997). To assess whether MIF/TKP can inhibit microglial activation in this model, MIF/TKP (750 μ M) or PBS were infused over the lesioned area, which was then examined at successive days post injury (dpi) to determine the extent of local microglial activation. Immunohistochemical staining against the microglial/macrophage marker Iba-1 (ionized calcium binding adaptor molecule-1) revealed that MIF/TKP treatment had a substantial effect on microglial morphology and cell numbers in comparison to the PBS control (Fig. 2). In sham-injured animals, microglia exhibited a ramified morphology indicative of a resting or non-activated state (Fig. 2A). SCI mice treated with PBS showed a temporal change both in the morphology and the number of microglia present in the lesioned area. Specifically at 3 dpi (not shown) and 7 dpi, many microglia with activated morphologies were found at the injury epicenter, while by 30 dpi the majority of the microglia had reverted back to resting state. In contrast, 14 days of administration of MIF/TKP reduced the number of microglia present and the majority (approximately 68%) of the microglia exhibited a ramified morphology. Treatment with MIF/TKP for only 7 days was less effective. To corroborate these results, quantitative immunoblotting was performed to examine Iba-1 protein levels in homogenates from the lesion epicenter. SCI dramatically increased Iba-1 levels in the PBS control group at 7 dpi (Fig. 2B). The levels of Iba-1 were decreased at 14 and 30 dpi but remained elevated over the sham control. Treatment with MIF/TKP blunted the rise in Iba-1 expression, again most effectively in the animals treated for 14 days (Figs. 2B and C). To determine whether MIF/TKP decreased microglia proliferation as opposed to recruitment, sections were immunostained against Iba-1 and the proliferation marker Ki67 (Fig. 2D). Quantification of Iba-1⁺/Ki67⁺ cells over the total number of Iba-1⁺ cells (proliferation index) indicated that MIF/TKP markedly reduced microglial proliferation during the first 1–2 weeks after injury (Fig. 2E). Together these results indicate that MIF/TKP is an effective inhibitor of microglial activation and proliferation following SCI, that it may be key to provide MIF/TKP soon after injury since the peak of microglial activation occurs during the first week, that a short period of treatment (7 days) is not as effective as a longer period (14 days), and that 14 days of treatment appears sufficient to prevent immune response activation.

Since MIF/TKP has been shown to reduce microglial-associated cytokine levels in *ex vivo* tissue culture settings (Rogove and Tsirka, 1998), we quantified cytokine production *in vivo* subsequent to SCI. The spinal cords of animals treated with PBS or MIF/TKP post injury were dissected to isolate three 3-mm segments corresponding to the lesion epicenter and the regions rostral and caudal to the lesion which were then homogenized and assayed for TNF- α . TNF- α levels in samples prepared from PBS-treated SCI mice were elevated at 1 dpi in all three segments in comparison to the non-injured (ni) sham control, and most dramatically in the lesion epicenter (Fig. 3). MIF/TKP treatment significantly decreased TNF- α production.

Inhibition of microglia activation alters the composition of the glial scar

Astrocytes are a major component of the glial scar that creates a barrier around the lesion epicenter. Since astrocytic activation follows and is promoted by the microglial response (Kreutzberg, 1996; Popovich et al., 1997), we sought to determine if astrogliosis was blunted following inhibition of microglial activation with MIF/TKP. GFAP

immunoreactivity was examined since GFAP expression is upregulated by astrocytes in the CNS following insult (Eddleston and Mucke, 1993; Ridet et al., 1997). GFAP immunoreactivity increased at the lesion border in PBS-treated SCI mice 7 dpi compared to the levels observed in the non-injured, sham-operated animal (Fig. 4A, arrows), and this increase was attenuated in MIF-treated mice. The change in GFAP protein levels was determined via quantitative immuno-blots, which confirmed lower GFAP levels at 7 dpi following MIF/TKP treatment (Figs. 4B and C).

To determine if the reduction in GFAP expression correlated with a decrease in astrocytic reactivity or a decline in astrocytic proliferation, Ki67⁺/GFAP⁺ cells were enumerated for each treatment (n=6/group) and astrocyte morphology analyzed. Astrocytes in PBS control groups appeared to be hypertrophic at 7 dpi (Fig. 4D) and exhibited enhanced proliferation (Fig. 4E). A difference in astrocytic morphology in the MIF/TPK-treated groups was noted particularly at 7 dpi, where many of the astrocytes retained a 'resting' morphology and the number of proliferative astrocytes was significantly decreased.

Inhibition of microglial activation reduces lesion volume

The lesion epicenter is devoid of astrocytes and oligodendrocytes, but contains a large number of macrophages and microglia that remain resident for months subsequent to injury (Wrathall et al., 1998). We accordingly speculated that MIF/TKP treatment might decrease the SCI lesion volume, since microglial/macrophage proliferation and recruitment along with astrocytic hypertrophy and proliferation were all reduced by this therapeutic approach. Lesion volumes were assessed by staining sections of the spinal cords with anti-GFAP to identify the lesion borders. The lesion volume, which was defined as the area devoid of GFAP signal (Fig. 4F), was found to be smaller when microglial/macrophage activation was inhibited, and this difference became statistically significant starting at 14 dpi (Fig. 4G).

Modulation of microglia/macrophage activation reduces levels of inhibitory chondroitin sulfate proteoglycans (CSPG)

We next examined levels of CSPGs, which are produced by reactive astrocytes and macrophages and are inhibitory to axonal regeneration following SCI (Bukhari et al., 2011). Spinal cord sections were immunostained using a pan-CSPG antibody (CS-56) and the mean fluorescent intensity was determined. Sham-injured groups exhibited little to no CSPG immunoreactivity (Fig. 5A). In PBS-treated mice, a significant increase in CSPG immunoreactivity was evident at the lesion epicenter extending out to areas bordering the lesion. In MIF-treated mice, in contrast, CS-56 immunoreactivity was significantly reduced at 7 and 14 dpi (Figs. 5A and B). Two CSPGs, neurocan and NG2, are particularly well known for having inhibitory effects on axonal regrowth; thus we directly examined their levels. PBS treatment subsequent to SCI resulted in an increase in neurocan levels peaking at 14 dpi and declining thereafter (Figs. 5C and D). Treatment with MIF/TKP for 14 days significantly reduced neurocan immunoreactivity at 14 dpi, and although other time points post injury showed trends towards lower levels of neurocan immunoreactivity, the decreases were not statistically significant. Similarly, NG2 immunoreactivity was elevated in PBS-treated groups at 7 dpi and then decreased over time (Figs. 5E and F). MIF/TKP treatment for 7 days reduced NG2 immunoreactivity starting at 7 days, while 14d MIF/TKP treatment reduced NG2 at 14 and 30 dpi.

Microglia/macrophages regulate oligodendrocyte survival

Approximately half of the oligodendrocytes in the peri-lesion area are thought to be lost within the first 2 days after SCI (Grossman et al., 2001; Lytle and Wrathall, 2007; Rabchevsky et al., 2007). Delayed and secondary oligodendrocyte death that occurs and expands outside the lesion area (Crowe et al., 1997) has been attributed to the actions of

activated macrophages, microglia and T-cells. Resting and activated microglia release glutamate, TNF- α , IL-10 and NOS, all of which contribute to oligodendrocyte cell death (Merrill and Scolding, 1999; Pineau and Lacroix, 2007; Rosenberg et al., 1999; Streit et al., 1998). These reports led us to investigate whether inhibition of microglia and macrophages with MIF/TKP affects T-cell and oligodendrocyte numbers in the peri-lesion area.

SCI induced an immediate decrease in the numbers of mature CC1⁺ oligodendrocytes in PBS-treated animals that slowly recovered over time (Figs. 6A and B). MIF/TKP treatment blunted the T-cell response (Supplemental Fig. 1) and oligodendrocyte loss with significant differences at 14 dpi and 30 dpi. Because secondary cell death associated with microglial/macrophage activation is considered to be apoptotic (Colton and Wilcock, 2010). TUNEL assays were performed to examine whether the increase noted in oligodendrocyte number in the mice treated with MIF/TKP was due to a decrease in oligodendrocyte cell death. In agreement with the literature, PBS control groups showed a significant increase in oligodendrocyte cell death over sham-treated animals at all time points observed (Figs. 6C and D). Inhibiting microglial/macrophage activation significantly reduced the number of TUNEL⁺ oligodendrocytes at 7 dpi ($p < 0.01$) and 14 dpi ($p < 0.05$).

To ascertain that microglial inhibition with MIF/TKP was directly associated with the decrease in oligodendrocyte death *in vivo*, primary microglia/oligodendrocyte co-cultures were prepared and treated with LPS, MIF/TKP, or MIF/TKP plus LPS (Figs. 6E and F). Treatment with MIF/TKP alone had no effect on oligodendrocyte death, as the number of TUNEL⁺ oligodendrocytes were similar to those observed in non-treated (NT) cultures. Oligodendrocytes expressing CNPase (marker for immature oligodendrocytes) were more prone to cell death, while MBP⁺ cells showed no significant difference after LPS treatment, indicating that immature oligodendrocytes are more susceptible to cell death induced by LPS-activated microglia. The addition of MIF/TKP along with LPS reduced the number of dying immature oligo-dendrocytes, indicating that inhibiting microglial activation with MIF/TKP suffices to reduce oligodendrocyte cell death (Figs. 6E and F).

NG2⁺ cell proliferation is enhanced following microglial suppression

NG2⁺ oligodendrocyte precursor cells (OPCs) proliferate and differentiate into mature myelinating cells to compensate for the loss of oligodendrocytes following trauma. Under normal physiological conditions OPC numbers are low, however following insult, OPCs proliferate rapidly over a time span from 48 h to 5 days (Levine, 1994). OPC numbers and proliferation were quantified following SCI in control and MIF/TKP-treated animals using NG2 and Ki67 immunofluorescent staining (Fig. 7). Microglial/macrophage inhibition (MIF/TKP treatment) resulted in significantly higher numbers of proliferating OPCs early after injury in comparison to PBS treatment (Figs. 7A and B). To address if activated microglia directly decrease OPC proliferation, primary OPC and microglial co-cultures were prepared and treated with LPS, MIF/TKP or MIF/TKP plus LPS and incubated with BrdU (Fig. 7C). No significant difference in OPC proliferation was observed when non-treated and MIF/TKP treated co-cultures were compared. LPS-treated co-cultures resulted in a decrease in OPC proliferation in comparison to controls (Fig. 7D). The addition of MIF/TKP to LPS-treated co-cultures increased the number of proliferating OPCs in comparison to LPS-treated cultures alone. These results suggest that activated microglia decrease OPC proliferative capacity following insult.

MIF/TKP treatment increases the number of myelinated axons present following spinal cord injury

The increased number of oligodendrocytes present following MIF/TKP treatment led us to examine whether myelin was preserved. Myelin basic protein (MBP), a component of

myelin and a marker of fully differentiated oligodendrocytes, was analyzed via quantitative immunoblotting of extracts from lesioned regions in SCI animals that had been treated with either PBS or MIF/TKP; a decrease in all three of the isoforms of MBP was observed (Figs. 8A–C). The 21.5 kDa isoform exhibited a profound loss in SCI animals treated with PBS and then a partial recovery by day 30 (Fig. 8B). In contrast, only modest loss of MBP was observed in SCI animals in which microglia/macrophage inhibition had been inhibited by treatment with MIF. Significant but less notable differences in loss of the MBP 17 kDa isoform were observed (Fig. 8C), and no significant differences were observed for the 18.5 and 14 kDa MBP isoforms. This is intriguing, since the 21.5 and 17 kDa isoforms are upregulated during myelination and remyelination, while the 18.5 and 14 kDa isoforms of MBP are associated with compact myelin (Capello et al., 1997). Spinal cord sections from PBS-treated animals revealed evidence of myelin debris within the lesion epicenter. In contrast, spinal cords of MIF/TKP-treated animals displayed faint myelin ‘tubes’ at 1 dpi which increased over time (Fig. 8D), indicative of the presence of myelin-wrapped axons.

To better visualize MBP organization in this setting, electron microscopic analysis was employed. Although demyelination was evident in both PBS and MIF/TKP sections at 3 dpi (Fig. 8E), MIF/TKP treatment resulted in a decreased number of demyelinated axons (Fig. 8F), a finding that persisted through 30 dpi. To determine the extent of myelination, myelin sheath thickness was analyzed by G-ratio analysis (the ratio of the myelinated axon diameter to the axon diameter). In this method of presentation, lower g-ratios indicate more extensive myelin wrapping of axons, which is what was observed as a consequence of MIF/TKP treatment 14 days after SCI (Fig. 8G). Hence, inhibition of microglia/macrophages with MIF/TKP is protective against demyelination and enhances subsequent remyelination.

Treatment with MIF/TKP reduces axonal dieback

Given that mature nerve cells are postmitotic, most of the functional recovery that may occur after injury is due to plasticity of the spared neurons. There are many molecules released or upregulated after SCI that inhibit the intrinsic growth capacity of axons, such as CSPGs and myelin-associated inhibitors (Fitch and Silver, 2008). Selected cellular interactions have also been reported to inhibit regeneration. Axonal dieback, a phenomenon where axons retract away from the lesion, has been associated with macrophage infiltration. An *in vitro* model of the glial scar provided evidence that macrophages interact with dystrophic axons and directly cause long-distance axonal retraction (Busch et al., 2009; Horn et al., 2008). Minocycline, a tetracycline derivative shown to inhibit microglia, reduces axonal dieback of cortical spinal tract (CST) axons, death of oligodendrocytes and improves functional recovery (Festoff et al., 2006; Stirling et al., 2004; Yune et al., 2007). Since more axons were viable around the approximate lesion epicenter as assessed by electron microscopy (Fig. 8E), we sought to determine if inhibition of microglia/macrophages with MIF/TKP would reduce dieback within the lesioned area. Retrograde axon labeling was performed by injecting CTB into the sciatic nerve 4 days prior to euthanizing the mice. MIF/TKP treatment for 7 and 14 days resulted in the presence of neurites within the lesioned area in comparison to the PBS control groups (Fig. 9A). The 14 day treatment groups exhibited the highest levels of CTB immunoreactivity within the lesion core. Higher magnification images revealed neurites extending in the glial scar (Fig. 9B) over longer distances in the MIF-treated mice compared to PBS- or 7d MIF-treated animals (Fig. 9C).

Discussion

We report here that inhibition of microglia/macrophages with the tripeptide MIF/TKP reduces astrogliosis, oligodendrocyte cell death and axonal dieback in the dorsal hemisection model of spinal cord injury (SCI). In this study, MIF/TKP was locally

administered at the lesion site via osmotic pump, where it diffused in a rostral–caudal manner with respect to the lesion, albeit the highest concentration of MIF/TKP was found within the lesion epicenter. We sought to determine the effect of suppression of microglial activation on the cellular and molecular environment.

Inhibition of microglial/macrophage activation with MIF/TKP notably reduced lesion volume at 14 dpi and 30 dpi, while at earlier time points the differences trended toward a decrease in lesion size rather than exhibiting significance. A higher dose of MIF/TKP might yield better results; alternately, microglia and macrophages may play a role in resolving the lesion volume at earlier phases that is inhibited by MIF/TKP. Nonetheless, preventing a large lesion volume in the early phase could be beneficial with respect to decreasing subsequent secondary damage.

We examined the extent of astrogliosis, as astrocytes are the principal cell type in the gliotic scar and their activation and accumulation is associated with microgliosis, which is thought to precede or complement the astrocytic reactivity (Giulian et al., 1989; Tanga et al., 2004). Although an astrocytic scar was present following MIF/TKP treatment, we found that the numbers, proliferation rate, and hypertrophy of the astrocytes were reduced. These findings were similar to results in a different setting obtained by Balasingam and Yong (1996), who reported that MIF/TKP significantly reduced astroglial reactivity following corticectomy. This decrease in astrogliosis may be due to a number of factors, since microglia secrete a myriad of cytokines and growth factors that in turn can act as triggers and modulators of astrogliosis. *In vitro* studies of microglia and astrocytic co-cultures have shown that microglial activation with LPS stimulates astrocyte proliferation. Blockade of prostaglandin E₂ using pharmacological inhibitors or via genetic knockout of microglial COX-2 has revealed that microglia secrete prostaglandin E₂ which enhances astrocyte proliferation (Zhang et al., 2009). Inhibition of microglia by MIF/TKP may have abrogated the expression of factors that are required for astrogliosis.

We then assessed CSPG expression. Although, neurocan is expressed primarily by neurons (Engel et al., 1996), it is also expressed by astrocytes *in vitro* (Asher et al., 2000; Oohira et al., 1994) and in various CNS injury models (Deguchi et al., 2005; Matsui et al., 2002; McKeon et al., 1999). During the duration of microglial/macrophage inhibition with MIF/TKP pan-CSPG expression, and specifically that of neurocan and NG2, were reduced. Following injury, macrophages, meningeal, Schwann and endothelial cells infiltrate the spinal cord and express NG2 (Jones et al., 2002; McTigue et al., 2006). We hypothesized that the observed decrease in NG2 was not due to inhibition of NG2⁺ OPCs, but rather to a decrease in NG2-expressing macrophages, since MIF/TKP affects all cells of monocytic origin. To assess whether the decrease in NG2 expression was due to a decrease in OPC population, NG2⁺ OPC numbers and proliferation were quantified. An increase in proliferating OPCs was observed at 1 dpi and no significant difference was obvious in OPC numbers following that time point *in vivo*. This is supported by previous literature reports demonstrating that macrophages influence the proliferation and differentiation of NG2⁺ progenitors (Schonberg et al., 2007). Co-cultures provided evidence that MIF/TKP inhibition of LPS treated microglia could attenuate the decline in OPC proliferation observed when microglia were treated with LPS alone. Our *in culture* data are consistent with the report that NG2⁺ cell proliferation is inhibited by the addition of purified OX42⁺ microglia/macrophages isolated from injured spinal cords (Wu et al., 2002), and with the data from Taylor in which TLR4 activated microglia decrease the proliferative properties of OPCs (Taylor et al., 2010).

Analysis of the mature oligodendrocyte population revealed an increase in oligodendrocyte numbers after MIF/TKP treatment, which might be due to the observed decrease in cell

death of oligodendrocytes and the higher number of OPCs found at 1 dpi that could differentiate and replenish the oligodendrocyte pool that is lost following SCI. It is plausible that the increase in oligodendrocyte number and survival are due to the reduction of factors secreted from microglia such as glutamate, TNF- α , IL-1 β and NOS, which contribute to oligodendrocyte cell death (Merrill and Scolding, 1999; Pineau and Lacroix, 2007; Streit et al., 1998). These results were also supported by *in vitro* co-cultures of oligodendrocytes and microglia. MBP isoform expression indicated that the 21.5 and 17 kDa isoforms associated with myelination were present at higher levels in MIF/TKP treated mice in comparison to PBS controls. This result is indicative either of remyelination, or, since the levels of the 21.5 kDa isoform in the MIF/TKP treatment never exceeded the levels observed in the sham injury, it is plausible that some demyelination was being prevented. MBP staining revealed that demyelination was occurring post injury in the MIF-treated animals, but not to the extent as that observed in PBS treated groups. New myelin formation was observed at later time points post injury suggestive of remyelination.

Since the oligodendrocyte numbers were higher and the number of oligodendrocytes undergoing cell death was decreased in the MIF/TKP-treatment groups in comparison to the PBS-treatment groups, it is conceivable that axons were not as dramatically demyelinated. Electron micrographs from 3 and 30 dpi support these findings: at 3 dpi demyelination was evident, higher numbers of small diameter axons remained myelinated when microglial/macrophage activation was inhibited, and micrographs of 30 dpi showed a significant increase in the number of myelinated axons.

A single oligodendrocyte is responsible for myelinating multiple axons. The loss of oligodendrocytes causes the demyelination of many spared axons, eventually causing distal axons to lose conduction capacity (Hains et al., 2003; Waxman, 2001). In addition to affecting axon conductance and signaling, oligodendrocytes also maintain the structural integrity of axons and contribute to neuronal survival (McTigue and Tripathi, 2008). The presence of more myelinated axons was accompanied by the higher density of axons within the lesioned area when microglia were inhibited with the tripeptide.

The observed increase in neurites extending into the glial scar found in MIF/TKP treatment groups could be explained by the reduction in oligodendrocyte cell death leading to sparing of myelin and prevention of axonal dieback. The reduction of astrocyte reactivity observed in the MIF/TKP treatment concomitantly reduced the expression of CSPGs, thereby creating a more permissive environment for axon growth, since CSPGs reduce the ability of axons to regenerate in areas of reactive gliosis (Davies et al., 1997, 1999; Hoke and Silver, 1996). All of these effects could be attributed to factors that microglia and macrophage secrete. TNF- α is mainly produced by cells of monocytic origin. Following CNS injury TNF- α is involved in mediating inflammation, neutrophil recruitment and apoptosis of glia thereby contributing to secondary damage. MIF/TKP inhibition of microglia and macrophages resulted in a significant reduction of TNF- α production both *in vitro* and *in vivo* following injury. TNF- α is associated with oligodendrocyte cell death in models of EAE: high concentrations of TNF- α evident in EAE affect sensitive oligodendrocytes and result in oligodendrocyte cell death (Akassoglou et al., 1998; Hovelmeyer et al., 2005; Jurewicz et al., 2003, 2005). Such a scenario could explain the presence of more neurites crossing through the glial scar.

In summary, we have shown that the overall prevailing role of microglia/macrophages following severe SCI is deleterious. Their detrimental effects can be effectively inhibited with the tripeptide, MIF/TKP, thereby reducing reactive gliosis and increasing the number of axons within the lesion epicenter. Since a dorsal hemisection is a severe insult, no behavior output was/can be assessed, as previously reported by other investigators (Cafferty et al., 2007). It would be interesting however to further these studies in a contusion model

with a combinatorial approach of other treatments that have been shown to ameliorate secondary injury or improve functional outcome, such as chondroitinase ABC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

The inhibitory properties of MIF/TKP are still effective after 14 days. A, Fluorescent images of primary microglia incubated with fresh 5 mM MIF/TKP, 14d MIF/TKP and/or LPS. B, The activation state of Iba-1⁺ microglia (red) was determined morphologically (microglia with less than 2 processes were considered activated, whereas microglia with 2 or more processes were considered resting) and via (C) TNF-α production. C and D, ELISA assays were performed on the microglial culture supernatant and (C) TNF-α and (D) IL-10 levels were measured. Error bars represent SEM, where *** $p < 0.001$, and ### $p < 0.001$ by ANOVA followed by Bonferroni's *post hoc* test (* comparison to untreated; # comparison to LPS). Scale bar, 50 μm (n=3 individual experiments). E, Biotinylated MIF/TKP was administered to mice via a micro-osmotic pump over the lesioned area of the spinal cord for 7 days and spinal cords were isolated at 7 dpi. Representative images of the distribution in biotinylated-MIF/TKP treated spinal cord. Scale bar, 100 μm. F, Higher magnification image of MIF/TKP. Scale bar, 50 μm.

Fig. 2. MIF/TKP inhibits microglial activation following dorsal hemisection spinal cord injury. A, Bright field images of spinal cord sections at 7, 14, and 30 days post injury (dpi) stained for the microglial marker Iba-1 (brown, detected with DAB; arrows point to individual microglia). Scale bar, 200 μ m. B, Representative experiment showing Iba-1 expression levels in the lesioned areas of spinal cords as analyzed by quantitative immunoblotting. C, Quantitative measurements of Iba-1 were performed using the Odyssey 2.1 software and the data was plotted as a ratio of the pixel volume of Iba-1 over the pixel volume of α -tubulin. D, Representative images of Iba-1 (green), the proliferation marker Ki67 (red), and nuclei (DAPI, blue) at 3 dpi (arrows pointing to Iba⁺/Ki67⁺ cells; scale bar, 50 μ m). E, Quantification of microglial proliferation. For statistical analysis, sham and MIF/TKP treated groups were compared to PBS controls for each time point evaluated. Error bars represent SEM, where * p <0.05, ** p <0.01, *** p <0.001 by ANOVA followed by Bonferroni's post-hoc test (n=6 individual experiments).

Fig. 3. Inhibition of microglia with MIF/TKP alters cytokine production post spinal cord injury. Spinal cords from PBS- and MIF/TKP-treated mice were cut into three 3 mm segments (rostral to the lesion, lesion epicenter, and caudal to the lesion), homogenized, and subjected to ELISA to quantitate TNF- α . Error bars represent SEM, where ** $p < 0.01$, and *** $p < 0.001$ by ANOVA followed by Bonferroni's *post hoc* test ($n = 3$ individual experiments); ni: non-injured.

Fig. 4. MIF/TKP inhibition of microglia reduces astrocyte reactivity and lesion volume. **A**, Fluorescent images of GFAP astrocytes in sagittal sections of spinal cords. Scale bar, 200 μm . **B**, Representative quantitative immunoblot of GFAP levels from spinal cord homogenates from PBS- and MIF/TKP-treated animals (arrows denote lesion epicenter bordered by astrocytes). **C**, Quantitative measurements of GFAP were performed using the Odyssey 2.1 software and the data plotted as a ratio of the pixel volume of GFAP over the pixel volume of α -tubulin. **D**, Representative images of spinal cords, isolated at 7 dpi from PBS and MIF/TKP treated mice, stained for GFAP (green) and the proliferation marker Ki67 (red) (arrows pointing to GFAP⁺/Ki67⁺ cells; scale bar, 50 μm). **E**, Quantitative analysis of proliferative astrocytes. **F**, Lesion volume (lesion area) was quantified from five sagittal sections by measuring the area devoid of stain surrounded by the GFAP⁺ astrocytic border (denoted by outlined area) and **G**, average values were obtained from 6 independent samples ($n=6/\text{time point/treatment}$) using NIH ImageJ software. Scale bar, 200 μm . Error bars represent SEM, where $*p<0.05$ by ANOVA followed by Bonferroni's post hoc test

(n=6 individual experiments). Statistical significance was analyzed between PBS-treated and MIF/TKP-treated mice for each time point.

Fig. 5. CSPG expression is reduced by MIF/TKP inhibition of microglia. Fluorescent images of spinal cords stained against A, the CSPG marker CS-56 (red), to determine the overall expression of CSPGs, C, neurocan and E, NG2. Scale bar, 200 μ m. Quantification of fluorescent intensity of B, CS-56s, D, neurocan and F, NG2 using ImageJ. Five 40 $\times$ fields were sampled and the optical density was measured for each. Statistical significance was analyzed between PBS-treated and MIF/TKP treated animals for each time point. Error bars represent SEM, where $*p < 0.05$ by ANOVA followed by Bonferroni's post hoc test ($n = 6$ individual experiments).

Fig. 6. Oligodendrocyte survival is directly affected by microglia following spinal cord injury. A and B, Spinal cord sections were stained for the mature oligodendrocyte marker, CC1 (red) and fluorescent images captured. A, Representative images of CC1⁺ oligodendrocytes in sham, PBS- and 14d MIF/TKP treated animals at 14 dpi scale bar, 20 μ m. B, Quantification of the number of CC1⁺ oligodendrocytes present following spinal cord injury in PBS- and MIF/TKP-treated spinal cords (# comparison to sham; * comparison to PBS treatments). C, TUNEL and CC1 staining of spinal cords and D, quantification of the percent of oligodendrocyte cell death (* comparison to PBS treated groups). Scale bar, 50 μ m. E and F, Confocal images of mixed microglial and oligodendrocyte co-cultures. E, Mixed cultures were treated with MIF, LPS, LPS and MIF/TKP, and F, oligodendrocyte cell death was assessed via TUNEL staining. The number of CNPase⁺/TUNEL⁺ cells was quantified (* comparison with nt; # comparison to LPS). Scale bar, 50 μ m. Error bars represent SEM,

where $*p < 0.05$, $^{\#}p < 0.01$, $***p < 0.001$, $###p < 0.001$ as determined by ANOVA followed by Bonferroni's post hoc test (n=6 individual experiments).

Fig. 7. Microglial inhibition increased OPC proliferation. A, NG2 immunostaining in the spinal cord to identify proliferative OPC as defined by co-staining with Ki67 and B, quantification of proliferation. Scale bar, 50 μ m. C, Confocal images of OPC and microglia co-cultures. Scale bar, 50 μ m. Co-cultures were treated with MIF, LPS, LPS and MIF/TKP; BrdU was added to assess D, OPC proliferation (*comparison with non-treated (NT); #comparison to LPS). Error bars represent SEM, where * $p < 0.05$, ** $p < 0.01$, ### $p < 0.001$ by ANOVA followed by Bonferroni's post hoc test (n=6 individual experiments).

Fig. 8.

More myelin is present with microglial inhibition. A, Quantitative immunoblotting was performed on spinal cord homogenates and MBP expression assessed; the 21.5 (B), and 17 kDa (C), isoform expressions were quantified. D, Fluorescent images of MBP bordering the lesion epicenter. Arrows point to myelin 'tubes'. Scale bar, 100 μ m. E, Representative electron micrographs of the dorsal penumbra of spinal cords treated with PBS and MIF/TKP for up to 14 dpi (scale bar: 2 μ m). Insets show high magnification images of unmyelinated axon (30 dpi PBS) and a myelinated axon (30 dpi MIF/TKP), arrow heads pointing to microtubules. F, Quantification of myelinated/unmyelinated axons from MIF/TKP and PBS treated animals, where 100 axons/group were counted. Error bars represent SEM, where * p <0.05, ** p <0.01, *** p <0.001 by ANOVA followed by Bonferroni's post hoc test (n =3 individual experiments). G, G-ratios of myelinated axons in control vs MIF/TKP-treated spinal cords following SCI.

Fig. 9. Enhancement of neurite outgrowth within the lesion core upon MIF/TKP treatment. **A**, Retrograde axon labeling with CTB via the sciatic nerve was performed on MIF/TKP- and PBS-treated mice and spinal cords isolated at 30 dpi (scale bar, 100 μm). **B**, High magnification images of CTB (red) and GFAP (green), dashed line denotes lesion border (scale bar, 50 μm). **C**, Neurite length was measured and plotted as distance from injury epicenter (0 μm). Error bars represent SEM, where $**p < 0.01$, $***p < 0.001$ by ANOVA followed by Bonferroni's post hoc test ($n = 6$ individual experiments).