Secondary Damage in the Spinal Cord after Motor Cortex Injury in Rats

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Abstract
When neurons within the motor cortex are fatally injured, their axons, many of which project into the spinal cord, undergo wallerian degeneration. Pathological processes occurring downstream of the cortical damage have not been extensively studied. We created a focal forelimb motor cortex injury in rats and found that axons from cell bodies located in the hindlimb motor cortex (spared by the cortical injury) become secondarily damaged in the spinal cord. To assess axonal degeneration in the spinal cord, we quantified silver staining in the corticospinal tract (CST) at 1 week and 4 weeks after the injury. We found a significant increase in silver deposition at the thoracic spinal cord level at 4 weeks compared to 1 week post-injury. At both time points, no degenerating neurons could be found in the hindlimb motor cortex. In a separate experiment, we showed that direct injury of neurons within the hindlimb motor cortex caused marked silver deposition in the thoracic CST at 1 week post-injury, and declined thereafter. Therefore, delayed axonal degeneration in the thoracic spinal cord after a focal forelimb motor cortex injury is indicative of secondary damage at the spinal cord level. Furthermore, immunolabeling of spinal cord sections showed that a local inflammatory response dominated by partially activated Iba-1-positive microglia is mounted in the CST, a viable mechanism to cause the observed secondary degeneration of fibers. In conclusion, we demonstrate that following motor cortex injury, wallerian degeneration of axons in the spinal cord leads to secondary damage, which is likely mediated by inflammatory processes.

Key words: spinal cord injury; stroke; traumatic brain injury; wallerian degeneration

Introduction
Motor cortex injury resulting from stroke or traumatic insults can have long-lasting and devastating effects on a patient’s health and quality of life. Unfortunately, treatment options for these conditions are still insufficient and of limited effectiveness. The majority of research directed at discovering promising strategies to limit tissue damage has been restricted to the brain, the primary location of the injury. However, axons of a substantial number of cortical neurons project into the spinal cord to form the corticospinal tract (CST), the major descending pathway for voluntary motor control in humans. The death of upper motor neurons in the cortex leads to wallerian degeneration (WD) of CST axons along the full length of their projections (Iizuka et al., 1989). Wallerian degeneration is a well-understood process that features recruitment and activation of immune cells (Vargas and Barres, 2007; Wang et al., 2009), thus a local immune reaction in the spinal cord following motor cortex injury is to be expected and has previously been reported in monkeys and rats (Basiri and Doucette, 2010; Nagamoto-Combs et al., 2007, 2010).

From animal models of spinal cord injury it is known that activated inflammatory cells residing around a spinal lesion can mediate a deteriorating process that damages initially spared tissue and thus contributes to secondary damage (Hagg and Oudega, 2006; Hausmann, 2003). For example, extensive production of cytotoxic substances and free radicals by activated inflammatory cells may injure spared axons neighboring lesioned axons within the CST, as well as myelinating oligodendrocytes. Thus, although spared axons are not directly affected by the primary lesion, cytotoxic inflammatory products may ultimately cause them to degenerate or become dysfunctional. Although secondary damage at the spinal level is a well-characterized feature of spinal cord injury, it is not known whether degenerative processes can exacerbate tissue damage in descending fiber tracts after motor cortex injury.

In this study, we tested the hypothesis that the inflammatory process accompanying WD of descending motor fibers...
facilitates secondary damage within spared CST fibers. To address this idea we assessed fiber degeneration and inflammation in the spinal cord at different time points following a focal devascularization lesion of the forelimb motor cortex. We found that fiber degeneration in the cervical spinal cord is already evident at 1 week following injury, whereas degeneration of hindlimb motor fibers whose cell bodies were spared by the lesion could only be seen at a delayed time point, suggesting a process of secondary damage. Early degeneration at the cervical level was accompanied by a local microglia response that might contribute to damage of initially spared fibers.

Methods

Animals and experimental groups

We used 42 male Sprague-Dawley rats (bioscience breeding colony at the University of Alberta) weighing 300–400 grams in two different experiments. For the first experiment, 24 rats were randomly assigned to four different experimental groups: forelimb motor cortex lesion and 1- or 4-week survival (n = 9 each), or sham surgery and 1- or 4-week survival (n = 3 each). For the second experiment, 18 rats received a hindlimb motor cortex devascularization lesion to study the time course of axonal degeneration in the thoracic spinal cord (survival at 1, 2, and 4 weeks post-injury, n = 6 each). The examiner was blinded to group identity. Procedures were performed in accordance with the Canadian Council on Animal Care, and were approved by the University of Alberta Animal Care and Use Committee for Biological Sciences.

Motor cortex lesion

Surgeries were performed under isoflurane anesthesia (2% isoflurane in 60% N2O and 40% O2). Body temperature was maintained at 37°C throughout the surgery. To create the cortical injury, a window was drilled into the skull above the forelimb area of the right motor cortex at the following coordinates relative to the bregma: anteroposterior (AP) +1 mm and mediolateral (ML) +1 mm, AP +1 mm and ML +4 mm, AP +4 mm and ML +4 mm, and AP +4 mm and ML +1 mm (Fig. 1B). Sham animals received a craniotomy by drilling a burr hole above the right forelimb motor cortex AP +1.5 mm and ML +1.5 mm. For the time course experiment, a cranial window 3×3 mm in size was created over the right hindlimb motor cortex: AP +0.5 mm and ML +0.5 mm, AP +0.5 mm and ML +3.5 mm, AP -2.5 mm and ML +0.5 mm, and AP -2.5 mm and ML +3.5 mm (Fig. 1D). Subsequently, devascularization of the entire exposed cortical area was achieved by removal of the dura, followed by pial stripping with a saline-soaked cotton swab (Kolb et al., 1997; Xue and Del Bigio, 2000).

Horizontal ladder

Animals receiving a lesion within the forelimb motor cortex were assessed on an elevated (20 cm) horizontal ladder before surgery and weekly after injury (Bolton et al., 2006). The rats had to cross a distance of 78 cm on rungs randomly spaced between 1 cm and 4.8 cm apart. Performances were recorded with a digital camera and analyzed frame-by-frame on a computer screen. An error was defined as a fall or a deep slip with the rat either losing balance, or the foot dropping below the rung to the point of the carpal/tarsal joint. The error rate was calculated by averaging the error score of three runs per testing session for each limb. The error rate is expressed as the percentage of erroneous steps out of the total number of steps taken to cross the ladder.

Perfusion and tissue collection

The animals were euthanized by an IP injection of 0.4 mL Euthanyl (Bimeda-MTC Animal Health Inc., Cambridge, Canada), and subsequently transcardially perfused with phosphate-buffered saline followed by phosphate-buffered 4% paraformaldehyde. The brains and spinal cords were collected and post-fixed in 4% paraformaldehyde at 4°C for 1–2 days, and were then cryoprotected in 30% sucrose solution for 2–4 days. The tissues were covered in OCT Tissue Tek (Sakura Finetek, Torrance, CA), frozen in methylbutane at −60°C, and stored at −80°C until sectioning. Cross sections of the brains were cut at 40 μm using a cryostat, and every fourth section was mounted on a gelatin-coated slide for lesion analysis. A caudal segment of the cervical enlargement (around C7), and a lower thoracic spinal segment (around T10), from each animal was cross sectioned at 25 μm for silver staining. Additionally, horizontal sections of the cervical enlargement and the thoracic region of the spinal cord were sectioned at 25 μm for Iba-1 immunostaining. All slides were stored at −20°C and warmed at 37°C for 1 h prior to staining to ensure proper attachment to the slides.

Histology

Cresyl violet staining. Brain sections were immersed in 70%, 95%, 95%, 100%, and 100% ethanol for 10 dips each, followed by 10 dips each in reverse order. After a 1-min wash in water, the slides were incubated in 0.25% cresyl violet in 200 mM acetate buffer for 5–7 min. Following 10 dips each in water, 70% and 100% ethanol, the tissue was differentiated in 0.25% glacial acetic acid until white matter was visible. Then the slides were dipped in 95% and 100% ethanol (10 dips each), incubated in two changes of HemoDe (1 min and 3 min), and cover-slipped using Permount (Fisher Scientific, Unionville, Canada).

Fluoro-Jade B staining. Coronal sections of the brains were stained with Fluoro-Jade B (Chemicon, Temecula, CA) to detect potential degeneration of neurons in the hindlimb motor cortex (Schmued and Hopkins, 2000). The slides were rinsed in distilled water (1 min), incubated in descending concentrations of ethanol (3 min in 100%, and 1 min each in 70% and 30%), followed by another rinse in water (1 min). They were then incubated in 0.001% Fluoro-Jade B in the dark for 30 min. Subsequently they were rinsed in distilled water three times for 1 min each. Before cover-slipping, the slides were immersed in ascending concentrations of ethanol (2 min each), and exposed to xylene two times for 2 min each. As a positive control we included in our staining trays an additional slide containing brain cross sections from animals that were exposed to global ischemia and killed 7 days after injury. This injury model produces selective neuronal death in the CA1 field of the hippocampus.

Silver staining. To identify degenerating axons, spinal cord cross sections were stained with a suppressive silver
method as previously described by Ye and colleagues (Ye et al., 2001). The slides were rinsed three times for 5 min each in distilled water, pretreated two times for 5 min each in a 4.5% NaOH and 0.6% NH₄NO₃ solution, and subsequently impregnated with silver nitrate for 10 min. After washing three times for 100 sec each in 0.5% Na₂CO₃, the slides were immersed in developing solution containing ethanol, paraformaldehyde, citric acid, and NH₄NO₃ at pH 5.8–6.1 for at least 1 min. After a short immersion in 0.9% saline, the tissue was finally fixed three times for 10 min each in 0.5% acetic acid, dehydrated through ascending concentrations of ethanol (2 min each), immersed two times for 2 min each in xylene, and cover-slipped using Permount.

Iba-1 immunohistochemistry. Horizontal spinal cord sections were rehydrated two times for 10 min each in TBS, followed by incubation with rabbit anti-rat Iba-1 primary antibody (1:1000, Wako Chemicals USA, Inc., Richmond, VA) overnight at 4°C (Kanazawa et al., 2002). After washing, a donkey anti-rabbit 488 fluorescent secondary antibody (1:500, Jackson ImmunoResearch West Grove, PA) was applied for 2 h. The slides were then incubated with DAPI (200 ng/mL).
for 30 min, washed, dehydrated through ascending concentrations of alcohol, and cover-slipped using Permount.

Histological analyses

Lesion assessment. For every animal receiving a lesion of the forelimb motor cortex, the caudal extent of tissue damage in the brain was assessed to detect potential overlap with the hindlimb motor cortex. In a rostral-caudal series of brain cross sections, the caudal end of the lesion was defined as the first section in which tissue was completely intact, showing no signs of cortical damage or hematoma formation. This section was then matched to the corresponding section in a stereotaxic atlas (Paxinos and Watson, 1998) to determine its anteroposterior location. The caudal end of the forelimb motor representation, and therefore the beginning of the cortical hindlimb area, was defined at 1.0 mm posterior to the bregma according to Zilles' stereotaxic atlas (Zilles, 1985).

Detection of neuronal degeneration in the hindlimb motor cortex. Coronal sections of the hindlimb motor cortex (12 sections/brain) were stained with Fluoro-Jade and examined under an epifluorescence microscope to identify degenerating neurons. The lesion extended into the most anterior sections, allowing us to examine the peri-infarct tissue. Neuronal degeneration was identified by either punctate labeling of the cell body or diffuse labeling of the neuropil.

Silver staining quantification. In spinal cord cross sections, photos of the CST contralateral to the brain injury were taken at 400× magnification. Using Adobe Photoshop 7.0, a square frame (62.5×62.5 μm) was drawn at a representative location in the CST projection for each section, and silver grains within this area were counted (Fig. 1E). Because silver staining quantifications were comparable (not statistically significantly different) in subsequent sections of one location for each individual animal (as analyzed initially in a sample of three animals), only one section per animal, location, and time point was quantified. Silver depositions in cervical spinal cord cross sections were counted to quantify degeneration of fibers originating in the forelimb motor cortex area affected by the primary cortical lesion. Silver staining in the thoracic spinal cord was assessed to detect potential degeneration of fibers originating in the hindlimb motor cortex whose cell bodies were spared by the cortical injury. Also, silver deposition at the thoracic level was compared between groups of 1- and 4-week survival to assess early versus delayed degeneration of axons.

Microglia quantification. Photos of the ventral part of the dorsal funiculus where the CST projects were taken at 200× magnification with a microscope. On a computer screen, 10 random photos of this CST region were analyzed (covering a distance of about 3.5 mm in total), and microglia cell counts for the left and right CST were individually summed for each animal. Only definable cell bodies were counted. As analysis was performed at about the same horizontal level through the dorsal CST in each animal, the variance of the CST projection in these horizontal sections did not differ much when one spinal level was analyzed. Because the CST gets narrower at the thoracic level, we only compared cervical or thoracic microglia numbers to counts taken at the same spinal level. The number of cells in the left, affected CST at one level was then compared between groups. In addition, we quantified the percentage of Iba-1-positive cells that were present in the left CST out of the total number of cells counted (sum of the left and right CST).

Microglia morphology. Using five of the randomly taken photos for microglia cell counting in the cervical spinal cord, two observers blinded to group identity individually rated microglia morphology to ascertain what stage of activation the microglia had reached (Stence et al., 2001). Whereas resident, quiescent microglia typically have long, thin processes, that build an arbor-like structure around the cell, partially activated or "primed" microglia, present with shorter, thicker processes with fewer branches, but a fluorescent signal more intense than that seen in the resting state (see Fig. 5E and F). Further, fully-activated microglia develop a phagocytic morphology (see Fig. 5G), and they become round, with few processes, and they have a strong signal following immunolabeling (Choo et al., 2008; Moisse and Strong, 2006). Based on these features, we rated the microglia in three categories: degree of branching, thickness of cell processes, and intensity of the fluorescent signal compared to background. A 3-point scoring system was used: 1 = low or thin, 2 = medium, and 3 = high or thick. Final scores represent the means of the two individual ratings.

Statistical analysis

All histological results were compared using unpaired t-tests, except for morphological scores for which the Mann-Whitney U test was employed. Horizontal ladder error rates were analyzed by repeated-measures analysis of variance (ANOVA). For all tests, a p value <0.05 was considered significant. All data are presented as mean ± standard error of the mean (SEM).

Results

Devascularization lesions were restricted to the forelimb motor cortex

Devascularization of a 9-mm² area above the right forelimb motor cortex produced V-shaped lesions that extended through all layers of the motor cortex (Fig. 1A; see Whishaw, 2000 for lesion characterization). Because devascularization lesions are known to expand beyond the actual devascularized area, we assessed the caudal extent of brain lesions at the time of euthanasia to control for potential overlap with the hindlimb motor cortex. The rostral border of the hindlimb motor cortex was defined at 1.0 mm posterior to the bregma, according to a stereotaxic atlas (Zilles, 1985). Although the caudal limit of the devascularization window was 1.0 mm anterior to the bregma (Fig. 1B), lesions expanded further, with their caudal limit ranging from 0.2 mm anterior to the bregma, up to 2.2 mm posterior to the bregma (Fig. 1C). Overall, devascularization resulted in a lesion restricted to the defined forelimb motor area (not further caudal than 1.0 mm posterior to the bregma) in 16 animals. We decided not to exclude the two animals whose brains showed hemorrhage within the ventricles beyond 1.0 mm posterior to the bregma at 1 week post-injury because a lack of silver staining in the thoracic spinal cord revealed that the hindlimb motor cortex
was intact in these animals (silver count of 1 each at the thoracic level, whereas the group mean was 9.8). The caudal lesion extent was not significantly different in animals that survived 1 week after injury (0.9 ± 0.3 mm posterior to the bregma), versus the 4-week survival group (0.5 ± 0.2 mm posterior to the bregma; \( p = 0.3 \)).

**Fibers originating from neurons spared by the cortical lesion undergo delayed degeneration in the spinal cord**

In silver-stained cross sections of the spinal cord, the degeneration of CST fibers was reflected by the deposition of black silver grains in the dorsal CST area, easily distinguishable from the brown background produced by the staining procedure (Fig. 1E). At 1 week after injury, silver deposits were abundant in the cervical CST contralateral to brain injury, and were not visible across the midline in any animal (52.3 ± 7.9 silver grains; Fig. 2A). In contrast to the cervical spinal cord, the thoracic cord showed markedly less axonal degeneration (9.8 ± 2.4 silver grains; Fig. 2B) at 1 week post-injury, values that were not statistically significantly different from those in sham rats (2.0 ± 1.0 silver grains). These findings confirm the expectation that axons originating from neurons within the damaged forelimb motor cortex undergo degeneration along their length. The relative lack of corticospinal axon degeneration in the thoracic spinal cord at this time point indicates that the hindlimb motor cortex was spared by the cortical lesion, and no substantial amount of secondary damage occurred in the early stages after injury. Interestingly, a significant increase in silver deposition was found in the thoracic CST region at 4 weeks post-injury (54.8 ± 18.5 silver grains; Fig. 2D), compared to 1 week post-injury (9.8 ± 2.4 silver grains; \( p = 0.028 \); Fig. 2B and E), indicating that fibers with cell bodies in the hindlimb motor cortex undergo

![Figure 2](https://www.liebertonline.com/neu)
delayed degeneration. Silver deposition was concentrated on
the affected side of the CST. However, a few silver grains
could be detected in the opposite side as well. At the same
time point, axonal degeneration at the cervical level was no
different from counts taken at 1 week post-injury (54.1 ± 13.2
silver grains at 4 weeks post-injury versus 52.3 ± 7.9 silver
grains at 1 week post-injury; Fig. 2A, C, and F).

**Neurons in the hindlimb motor cortex do not degenerate following forelimb motor cortex injury**

To detect potential degeneration of neurons within the
hindlimb motor cortex, we searched for Fluoro-Jade-positive
cells in coronal sections directly caudal to the forelimb
motor cortex lesion. In 8 out of 9 animals that survived 1
week post-injury no labeled cells were found in the region
of interest (Fig. 3A). Only one animal showed Fluoro-Jade
labeling within the hindlimb motor cortex. Because the
survival time for this rat was 1 week, we did not exclude it
from the study. Most important for our experiment is that
no Fluoro-Jade labeling could be found in the hindlimb
motor cortex for any animal at 4 weeks after injury (Fig.
3A). Bright staining in positive control sections (the CA1
region following global ischemia) confirmed that our
staining protocol worked (Fig. 3B).

**After injury to the hindlimb motor cortex, corticospinal
tract axons undergo non-delayed degeneration
in the thoracic spinal cord**

The increase in silver staining in the CST at the thoracic
level at 4 weeks following injury to the forelimb motor cortex
can be interpreted as secondary damage. Alternatively, this 4-
week delay might be necessary for axonal degeneration to
occur at a location more distant from the primarily injured
cell body. To exclude this possibility we examined the time course
of fiber degeneration in the thoracic CST after a hindlimb
motor cortex injury. Following a direct injury, the peak of
silver deposition in the thoracic CST was already observed at
1 week post-injury, with 202.3 ± 17.5 silver grains (Fig. 4A),
followed by a significant decline to 94.5 ± 23.2 at 2 weeks post-
injury ($P = 0.0043$; Fig. 4B) and a minor drop in number of
silver grains at 4 weeks post-injury (76.5 ± 8.7; Fig. 4C). Thus,
if directly injured, the majority of degenerating fibers are
present in the thoracic spinal cord as early as 7 days after
injury, and their numbers decline thereafter (Fig. 4D). This
time course of degeneration confirms that late onset of tho-
racic fiber degeneration after a lesion restricted to the forelimb
motor cortex is indicative of secondary damage, and is not
due to primarily-injured hindlimb motor neurons at the cor-
tical level.

**Recruitment and activation of microglia supports
the hypothesis of inflammation-induced secondary
damage in the spinal cord**

In order to detect recruitment of microglia cells within the
cervical dorsal CST at 1 week following forelimb motor cortex
injury, we compared the number of Iba-1-labeled cells (a
marker for microglia and macrophages) present in the left
CST (contralateral to the cortical lesion) between lesioned and
sham rats. A significant increase ($p = 0.016$) in the number of
microglia cells in the CST occurred in injured (119.4 ± 9.4)
compared to sham animals (70.7 ± 4.7; Fig. 5A). Interestingly,
Iba-1-labeled cells seemed to be highly concentrated in the
affected side of the CST, the same region found to undergo
axonal degeneration using the silver stain (see Fig. 2A). In-
deed, a quantitative comparison revealed a significant in-
crease in relative microglia presence in the affected side of
the cervical CST (65.8 ± 1.8% on left side) in lesioned animals
compared to sham animals (49.6 ± 5.7% on the left side;
$p = 0.0039$; Fig. 5C and D).

A comparison of microglia cell counts in the left thoracic
CST revealed no increase in microglia presence between 1
week (75.7 ± 9.5) and 4 weeks post-injury (81.3 ± 5.1, Fig. 5B).
These values were comparable to those of sham rats (75.7 ±
11.8 at 1 week, and 61.33 ± 15.7 at 4 weeks). Also, a concen-
tration of cells in the affected side of the CST could not be
found at the thoracic level; instead the cells were distributed

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**FIG. 3.** Fluoro-Jade B staining of the hindlimb motor cortex following a forelimb motor cortex lesion. (A) Background
staining without positive Fluoro-Jade signal representative of
17 out of 18 lesioned rats, indicating that there is no
observable neuronal death in peri-infarct hindlimb motor
cortex. (B) A positive control slide shows consistent Fluoro-
Jade labeling of CA1 cells, as is expected following global
ischemia. (Color image is available online at www.liebert
online.com/neu).
equally on both sides. We therefore conclude that a local inflammatory response had not yet been mounted in the thoracic CST at 4 weeks post-injury.

Morphological assessment of cells present in the cervical CST after motor cortex injury revealed a tendency toward thicker processes, an increase in signal intensity, and less branching, than in the cells seen in sham animals (Fig. 5H–J). However, only the difference in process thickness was found to be statistically significant ($p = 0.02$). Based on the known morphological features of microglia during activation (Fig. 5E–G), these findings indicate that microglia cells within the cervical CST are in a stage of activation.

Delayed degeneration of axons with cell bodies in the uninjured hindlimb motor cortex is not accompanied by changes in hindlimb function

When scored on the horizontal ladder, all devascularized animals showed a significant increase in error rate of the left forelimb at 1 week post-injury ($p = 0.006$; $27.7 \pm 4.0\%$ versus $0.9 \pm 0.6\%$ at baseline; Fig. 6A). Furthermore, spontaneous recovery over time was also observed. A repeated-measures ANOVA of forelimb error rate at week 1 and week 4 indicated a significant time $\times$ group interaction ($p = 0.024$) due to the improvement in the lesioned group ($10.8 \pm 1.8\%$ at 4 weeks post-injury). In contrast, error rates for the left hindlimb did not show a group difference, as neither the group ($p = 0.68$) nor the group $\times$ time interaction was significant ($p = 0.84$; Fig. 6B). Thus, the histological finding of degenerating corticospinal axons at the thoracic level was not reflected by a decrease in hindlimb performance on the horizontal ladder.

Discussion

In the present study, we demonstrate that following motor cortex injury, axons with cell bodies spared by the primary cortical lesion incorporate significant silver labeling at a delayed time point. This suggests delayed fiber degeneration of tracts not directly affected by the cortical lesion. In our rat model of forelimb motor cortex injury, the number of degenerating silver-stained axons in the thoracic spinal cord increased dramatically between 1 and 4 weeks post-injury. Furthermore, we showed that this delay in degeneration cannot be explained by degenerative processes taking longer to reach the thoracic versus the cervical spinal cord. Neither is the delayed nature of degeneration likely to be the result of delayed motor neuron degeneration in the hindlimb motor cortex, as there were no Fluoro-Jade-labeled cells in this region at 1 or 4 weeks post-injury. We therefore conclude that the observed delayed axonal degeneration of thoracic CST fibers is evidence of secondary damage at the spinal cord level. In addition, we found that
walleric degeneration of primarily-injured axons in the cervical CST at 1 week post-injury was accompanied by a marked increase in local microglia numbers, and that these microglia showed the morphological characteristics of partial activation or “priming.” This early inflammatory response in the spinal cord constitutes a feasible mechanism for mediating secondary damage remote from the primary injury site.

Although there is a wide variety of animal models for cortical injury in general and ischemia in particular, the
options for producing a focal injury are relatively few. We chose devascularization because it is a well-established method for generating ischemic lesions that reliably damage all layers of the cortex in a defined area (Whishaw, 2000). In addition, the consistency in lesion volume and location is far superior to other forms of ischemic injury, such as middle cerebral artery occlusion or endothelin injection (Windle et al., 2006; Zhao et al., 2008).

The results of the silver stain quantification raise various questions. For example it is unclear why our results show that the amount of silver deposition in the cervical CST region of injured animals at 1 week is comparable to that observed at 4 weeks after injury. It seems surprising that in this scenario the number of degenerating axons does not decline over time, as the time course experiment clearly demonstrated a decline over the weeks following injury. This could possibly be explained by secondary damage to fibers originating in the hindlimb motor cortex, which at 4 weeks post-lesion are also detected in the cervical enlargement. Thus the decline in silver labeling of primarily-injured axons likely parallels an increase in silver labeling from more recent degeneration of fibers originating in the hindlimb motor cortex.

Another unanticipated finding is that following direct injury to the hindlimb motor cortex, there is more deposition of silver in the thoracic CST than in the cervical CST following lesioning of the forelimb motor cortex. There are two possible explanations for this difference. First, the time course experiment indicates that there is a peak of silver deposition, followed by a decline over time. The 1-week time point may be past this peak at the cervical level. Second, it is important to note that the lesion sizes between studies (time course and secondary degeneration) are not necessarily comparable. These were two different sets of experiments performed by different experimenters.

The most important conclusion we can draw from silver staining in this study is that fibers whose cell bodies are spared by a primary cortex lesion degenerate at a delayed time point in the spinal cord. This fact motivates us to search for potential mechanisms that cause secondary damage, and thereby constitute a link between the primary injury and secondary processes. It has previously been reported that following the death of motor cortex neurons, their axons undergo wallerian degeneration along their projections into the spinal cord (Iizuka et al., 1989), thereby initiating an inflammatory response (Basiri and Doucette, 2010; Fu et al., 2004; Moisse et al., 2008; Nagamoto-Combs et al., 2007, 2010). Inflammation in the spinal cord, however, is a highly complex process, and its benefits and disadvantages remain controversial (Bethea and Dietrich, 2002; Crutcher et al., 2006; Hausmann, 2003; Schwartz, 2003; Schwartz et al., 1999). It has been suggested that the inflammatory process may contribute to a favorable environment for regeneration of severed axons (Previtt et al., 1997; Rapalino et al., 1998). On the other hand, activated inflammatory cells may facilitate secondary damage after spinal cord injury by releasing proinflammatory cytokines, and potentially cytotoxic substances such as reactive oxygen species and nitric oxide (Bethea and Dietrich, 2002; Hagg and Oudega, 2006; Hausmann, 2003). The present results suggest that similar detrimental events occur in the spinal cord following motor cortex injury, which is likely due to inflammation in the spinal cord evoked by axons undergoing wallerian degeneration. We found an increase in microglia presence in the cervical spinal cord at 1 week post-injury, that was localized to the area of axonal degeneration as shown by silver staining. However, when the contralateral and ipsilateral CST microglia numbers were compared, no statistically significant difference was found between groups. This points towards a local recruitment and redistribution of inflammatory cells rather than a global increase. Counter-intuitively, no increase in microglia numbers was found in the thoracic spinal cord accompanying axonal degeneration/positive silver stain at 4 weeks post-injury, as the microglia numbers were no different from those seen in sham rats or injured rats at 1 week post-injury. This could possibly be explained by the time point following lesioning, which captured the onset of secondary fiber degeneration, but missed the subsequent recruitment of microglia. The cervical spinal cords...
of animals that survived 4 weeks after injury were not analyzed for microglia numbers, as this would not address the hypothesis of secondary damage. However, similar values to what we found cervically at 1 week post-injury are to be expected, because previous reports have shown that microglia remain around damaged tissue sites for weeks (Basiri and Doucette, 2010).

When interpreting numbers of inflammatory cells at sites of tissue injury, we have to keep in mind that there is one major difference between damage developing in the spinal cord after motor cortex injury and that seen after direct spinal cord injury: after direct spinal trauma, the blood–brain barrier (BBB) is locally disrupted, and blood-borne immune cells are recruited into the injured nervous tissue (Schnell et al., 1999). BBB breakdown gradually declines with increasing distance from the primary lesion, even though axonal degeneration is occurring remote from this site (Schnell et al., 1999). Therefore, BBB disruption at the spinal cord level following cortical damage is probably negligible, leaving microglia rather than blood-borne macrophages and T cells to constitute the majority of activated immune cells. This rationale was confirmed in the present study by the scarcity of macrophage-like amoeboid cells among Iba-1-labeled cells. Instead, we observed a predominance of partially-activated microglia cells that stands in agreement with previous reports of immune cell profiles within the spinal cord remote from the primary injury site (Koshinaga and Whittemore, 1995; Moisise et al., 2008; Shi et al., 2009). Our results indicate that these innate immune cells are capable of mediating a remarkable amount of secondary damage at locations where the BBB is presumably intact. In fact, activated microglia have been shown to release pro- as well as anti-inflammatory cytokines, and appear to be well suited to facilitate tissue damage via cytotoxic metabolites (Moisise and Strong, 2006).

In addition to elucidating the interplay of potential mechanisms, one major concern to be addressed in the future will be the functional relevance of fiber loss resulting from secondary damage. We chose the horizontal ladder test as a paradigm to examine hindlimb motor performance. This test has been shown to be sensitive to CST damage (Bolton et al., 2006). In spite of the fiber damage observed in the left thoracic CST at 4 weeks post injury, there was no evident decrease in motor performance of the left hindlimb on the horizontal ladder. However, this lack of measurable motor deficits produced by secondary damage has been reported previously (Czeiter et al., 2008; Iizuka et al., 1989). Also, in a clinical setting, spinal cord–injured patients, as well as stroke patients, usually recover gradually, although secondary tissue damage is known to occur in a delayed fashion (Ishida and Tominaga, 2002). One explanation for this observation is that slowly developing pathological processes like the spread of damage to initially spared fibers are paralleled by abating of the acute injury responses and repair mechanisms initiated by the primary injury. Deficits in function due to secondary damage might thereby be masked in motor tests, due to the ongoing recovery and compensation that follow the injury. Hence the question as to what amount of secondary damage leads to assessable functional deficits, or more likely prolonged recovery, in animal models of motor cortex injury remains unanswered so far.

Nevertheless, the present study is the first to present evidence of secondary damage at the spinal cord level after motor cortex injury, and thus introduces a pathological role for the spinal cord in conditions like stroke and traumatic brain injury. Moreover, these novel insights will shed new light on present-day and potential clinical treatment strategies for patients. Currently, the role of inflammation in the brain after stroke, for example, remains controversial (Dinagl, 2004). Our results encourage therapeutic intervention at the level of the spinal cord for a prolonged time period. However, anti-inflammatory treatments should be carefully designed to establish the required delicate balance of the inflammatory processes seen after CNS injury. Further research is certainly needed to confirm and expand our current knowledge of secondary damage after motor cortex injury, specifically to identify other potential mechanisms behind secondary axonal degeneration, as well as its functional relevance and its responsiveness to treatment.

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