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Sprouting of axonal collaterals after spinal cord injury is prevented by delayed axonal degeneration

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ABSTRACT

After an incomplete spinal cord injury (SCI), partial recovery of locomotion is accomplished with time. Previous studies have established a functional link between extension of axon collaterals from spared spinal tracts and locomotor recovery after SCI, but the tissular signals triggering collateral sprouting have not been identified. Here, we investigated whether axonal degeneration after SCI contributes to the sprouting of collaterals from axons spared after injury. To this end, we evaluated collateral sprouting from BDA-labeled uninjured corticospinal axons after spinal cord hemisection (SCI^H) in wild type (WT) mouse and Wld^S mouse strains, which shows a significant delay in Wallerian degeneration after injury. After SCI^H, spared fibers of WT mice extend collateral sprouts to both intact and denervated sides of the spinal cord distant from the injury site. On the contrary, in the Wld^S mice collateral sprouting from spared fibers was greatly reduced after SCI^H. Consistent with a role for collateral sprouting in functional recovery after SCI, locomotor recovery after SCI^H was impaired in Wld^S mice compared to WT animals. In conclusion, our results identify axonal degeneration as one of the triggers for collateral sprouting from the contralesional uninjured fibers after an SCI^H. These results open the path for identifying molecular signals associated with tissular changes after SCI that promotes collateral sprouting and functional recovery.

Introduction

Injuries to the spinal cord represent a significant clinical problem leading to lifelong disabilities with an enormous social and economical impact (Ackery et al., 2004; Hendriks et al., 2006; Thuret et al., 2006). Despite the poor regenerative capabilities of central nervous system (CNS) neurons, spontaneous functional recovery has been reported after incomplete spinal lesions in various organisms, including rats (Ballermann and Fouad, 2006; Bareyre et al., 2004; van den Brand et al., 2012), mice (Courtine et al., 2008; Liu et al., 2010; Valenzuela et al., 2012; Yip et al., 2010), cats (Martinez et al., 2011; Rossignol et al., 2002), monkeys (Babu and Namasivayam, 2008; Courtine et al., 2005) and humans (Raineteau and Schwab, 2001). A functional association between locomotor recovery after spinal cord injury (SCI) and collateral sprouting from intact spinal cord axons has been proposed. In this respect, it has been shown that axon collaterals generate a new intraspinal circuit that bypass the injury zone and promotes locomotor recovery (Ballermann and Fouad, 2006; Bareyre et al., 2004; Courtine et al., 2008; Rossignol and Frigon, 2011). Collateral sprouting is not restricted to spinal cord levels close to the lesion (Ballermann and Fouad, 2006), suggesting that signals that propagates along the spine trigger axonal extension from intact neurons.

Several studies have been carried out in order to identify activators of collateral extension after spinal cord and brain damage. Among the interventions proved to enhance collateral sprouting are the upregulation of neurotrophic factors such as NT3 and BDNF (Fouad et al., 2010; Ueno et al., 2012; Zhou et al., 2003), inhibition of myelin associated proteins (Bareyre et al., 2002; Lee et al., 2010), enzymatic digestion of the glial scar (Barrit et al., 2006; Wang et al., 2011), activation of the immune response and release of cytokines (Chen et al., 2008; Oshima et al., 2009), and activation of different molecular pathways such as Rho/ROCK (Chan et al., 2005), mTOR (Liu et al., 2010), STAT3 (Bareyre et al., 2011) and NCS1 (Yip et al., 2010). Although these tissular manipulations represent attractive therapeutic strategies to enhance collateral sprouting, the endogenous mechanism underlying the spontaneous extension of collateral after nervous system damage is still unknown.

SCI elicit a plethora of cellular, tissular and systemic reactions with a defined temporal and spatial sequence, including the degeneration of axons severed from their cell body by a mechanism known as Wallerian

Abbreviations: SCI, spinal cord injury; SCI^H, spinal cord hemisection; Wld^S, Wallerian degeneration slow mutant mice; IL, ipsilateral to the injury; CL, contralateral to the injury; VST, vestibulospinal tract; VLAT, ventrolateral spinal tract.

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degeneration (Profyris et al., 2004; Willerth and Sakiyama-Elbert, 2008), changes in neurotrophin expression and its receptors (Hajebrahimi et al., 2008), inflammatory reactions along the spinal cord (David and Kroner, 2011; Donnelly and Popovich, 2007), apoptotic death of neurons and oligodendrocytes (Beattie et al., 2002) and formation of a glial scar at the injury region (Yiu and He, 2006).

Wallerian degeneration has been shown to evoke growth responses from damaged as well as intact neurons (Court and Alvarez, 2000; Diaz and Pecot-Dechavassine, 1990; Kerschensteiner et al., 2004; Ramer et al., 1997). Axonal degeneration takes place by mechanisms associated to mitochondrial dysfunction (Barrientos et al., 2011), intra-axonal calcium rise and calpain activation (Schlaepfer and Bunge, 1973). In wild-type mice, axonal injury triggers degeneration of severed axons by two to three days (Court and Coleman, 2012). Nevertheless, in the Wld^S (Wallerian degeneration-slow) mice, Wallerian degeneration after injury is delayed by weeks in both the CNS and the PNS (Coleman, 2005). It has been reported that after an SCI, the Wld^S mice show delayed locomotor recovery (Zhang et al., 1998) and diminished autonomic dysreflexia (Jacob et al., 2003). Interestingly, changes in the extent of collateral sprouting from spared fibers after SCI might be underlying those behavioral responses in the Wld^S mice.

Here we asked if following SCI, Wallerian degeneration of axons triggers collateral sprouting from contralesional uninjured axons and the functional association between this growth response and locomotor recovery. By using a genetic model of delayed axonal degeneration (Wld^S) we demonstrate that after partial spinal damage, collateral sprouting of uninjured corticospinal neurons and locomotor recovery is dependent on axonal degeneration, defining for the first time an intrinsic signal triggering plastic responses in the spinal cord after damage.

Materials and methods

Animals

Adult (10–12 weeks) female and male mice were obtained from the Animal Facility of the Biological Sciences Faculty of the P. Catholic University of Chile. The mouse strains used were C57BL/6 (WT) and Wallerian degeneration slow mutant mice (C57BL/OlaHsd-Wld^S; Lunn et al., 1989). The experiments were approved by the P. Catholic University bioethics committee (DFCB-078/2008).

The total number of animals used in this study was 42 WT (23 for anatomy, 3 for in vitro studies, 8 for locomotion and 8 excluded) and 50 Wld^S (24 for anatomy, 3 for in vitro studies, 10 for locomotion and 13 excluded). The animals excluded from this study matched one of these criteria: complete paralysis of hindlimbs or movement of the right hindlimb one day after SCI^H surgery (WT: 8 Wld^S: 4) and animals who died before end of study due to natural causes or euthanasia due to severe distress (WT:0; Wld^S: 9).

Surgical procedures

Mice were anesthetized with a single dose of 330 mg/kg I.P. of 2-2-2 Tribromoethanol (Sigma, St. Louis, MO, USA). Animals received laminectomies of the dorsal half of the thirteenth thoracic vertebra (T13) corresponding to L3 spinal level. This was followed by a lateral dorso-ventral hemisection of the right side of the spine (SCI^H) using a pair of vannas microscissors (RS-5658, ROBOZ, Gaithersburg, MD, USA) as previously described (Valenzuela et al., 2012). The bone removed by laminectomy was replaced with Gelita-Spon (Gelita Medical, Ederbach, Germany), and the wound was closed by suturing the muscle with 4-0 silk and the skin with mice wound clips. Sham-operated animals include the removal of the vertebra without spinal hemisection. Control animals underwent only stereotaxic surgery but no laminectomy intervention. For post-operative recovery, mice were placed in a temperature-controlled chamber until fully awake. The animals did not show evidence of bladder malfunction. For collateral quantification, the animals were allowed to survive for 7 or 35 days. For behavioral analysis, the animals were allowed to survive for 35 days.

Corticospinal tract (CST) tracing

To anterogradely label the corticospinal tract (CST) fibers mice were injected with BDA MW 10.000 (Invitrogen, Carlsbad, CA, USA) into the right hindlimb sensorimotor cortex, as previously described (Tysseling-Mattiace et al., 2008). Briefly, mice were anesthetized with 2-2-2 Tribromoethanol (Sigma, St. Louis, MO, USA), placed in a stereotaxic frame and injected in three points as follows: −1.0 mm lateral to the midline, at 0.5 mm, −0.5 mm and −1.0 mm from Bregma, and at a depth of 0.7 mm ventral to the dura, using a 5 μl Hamilton syringe fitted with a 34 G needle. Each injection delivered 0.5 μl of the tracer over a 2 min period and the syringe was left in the site for 4 min before removal. The tracer was injected fourteen days before sacrificing the animals.

Tissue preparation

Animals were transcardially perfused with ice-cold 4% paraformaldehyde in 0.1 M PBS, pH 7.2–7.4. The spinal cords were dissected and post-fixed for 3 h in 4% paraformaldehyde, subjected to a sucrose gradient (10, 20 and 30% sucrose in PBS) and fast frozen in OCT (Tissue-Tek, Alphen aan den Rijn, The Netherlands) using liquid nitrogen. A 4 mm block containing the cervical enlargement (C3–C5) region or the lumbar enlargement (L3–L5) region was transversally sectioned at 20 μm using a cryostat microtome (Leica, Nussloch, Germany) and direct mounted in Superfrost slides (Fisherbrand Superfrost Plus, Thermo Fischer Scientific, USA).

Collateral sprouting quantification

To visualize the BDA-labeled CST collateral sprouting axons, sections were washed 3 times in 0.1 M PBS, incubated for 2 h in Alexa-488 conjugated Avidin (1:250, Invitrogen, Carlsbad, CA, USA) in 0.1% Triton X-100, 5% gelatin from cold water fish (Sigma, St. Louis, MO, USA) in PBS, washed three times in 0.1 M PBS and covered with Fluoro-mount (Electron Microscopy Sciences, Hatfield, PA, USA). Slices were imaged using an OLYMPUS IX71 microscope fitted with a CCD camera. Three transverse sections per animal at either the cervical or lumbar spinal level were selected for analysis. In each section, two regions of interest were defined: contralateral to the injury (contralateral horn, CL), containing the fibers growing towards the uninjured side of the spinal cord (left in figures and images); and ipsilateral to the injury (ipsilateral horn, IL), containing the fibers growing towards the injured side of the spinal cord (right in figures and images) (Fig. 1A). To diminish the background induced error in the quantification of collaterals a hand-made digital outline of collaterals was drawn by an experimenter blind to the treatment and mice strain (Fig. 1B). The digital outline was drawn in Photoshop CS2 (Adobe 9.0.2) with a 4 pixel wide pencil at 120% magnification images. The collateral sprout profile was calculated using a custom made script for MATLAB software version 7.0.1 (MathWorks). Briefly, the procedure selects a 1-pixel (3.6 μm) wide column in the y-axis of the outline mask and sums the red pixels, generating the number of pixels per column, and repeats the procedure along the entire outline mask. The collateral sprout profile is the plot of the number of pixels per column along the x-axis. By doing this, we obtained a 2D profile with information about the magnitude and location of the collateral population (Fig. 1C). To correct for the inter-animal variation in the tracing efficiency, we normalized the collateral profile matrix in each slide by the number of traced fibers in the main CST of the same slide, obtaining the collateral pixels per stained CST fibers.