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A CHARACTERIZATION OF WHITE MATTER PATHOLOGY FOLLOWING SPINAL CORD COMPRESSION INJURY IN THE RAT

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Abstract—Our laboratory has previously described the characteristics of neuronal injury in a rat compression model of spinal cord injury (SCI), focussing on the impact of this injury on the gray matter. However, white matter damage is known to play a critical role in functional outcome following injury. Therefore, in the present study, we used immunohistochemistry and electron microscopy to examine the alterations to the white matter that are initiated by compression SCI applied at T12 vertebral level. A significant loss of axonal and dendritic cytoskeletal proteins was observed at the injury epicenter within 1 day of injury. This was accompanied by axonal dysfunction, as demonstrated by the accumulation of β-amyloid precursor protein (β-APP), with a peak at 3 days post-SCI. A similar, acute loss of cytoskeletal proteins was observed up to 5 mm away from the injury epicenter and was particularly evident rostral to the lesion site, whereas β-APP accumulation was prominent in tracts proximal to the injury. Early myelin loss was confirmed by myelin basic protein (MBP) immunostaining and by electron microscopy, which also highlighted the infiltration of inflammatory and red blood cells. However, 6 weeks after injury, areas of new Schwann cell and oligodendrocyte myelination were observed. This study demonstrates that substantial white matter damage occurs following compression SCI in the rat. Moreover, the loss of cytoskeletal proteins and accumulation of β-APP up to 5 mm away from the lesion site within 1 day of injury indicates the rapid manner in which the axonal damage extends in the rostro-caudal axis. This is likely due to both Wallerian degeneration and spread of secondary cell death, with the latter affecting axons both proximal and distal to the injury. © 2013 Published by Elsevier Ltd. on behalf of IBRO.

Key words: spinal cord injury, axons, demyelination, neurofilament, compression injury, white matter.

INTRODUCTION

Despite recent advances in pre-clinical research (Baptiste and Fehlings, 2007; Kwon et al., 2011), spinal cord injury (SCI) still results in permanent and devastating disabilities for which there are currently no effective pharmacological interventions. SCI is characterized by an initial injury which leads to a cascade of secondary injury processes that spread away from the injury epicenter, affecting neurons, glia and *en passant* axons (Priestley et al., 2012). Previous groups have examined the spatiotemporal development of neuronal loss. Using contusion models of SCI, they have demonstrated that secondary degeneration develops over hours, weeks and even months following injury in both rats and primates (Crowe et al., 1997; Liu et al., 1997; Grossman et al., 2001). Previously, our laboratory has studied the spatiotemporal pattern of gray matter pathology, in rat (Huang et al., 2007) and mouse (Lim et al., 2013) compression models of SCI. These experimental models have been reported to reproduce the hypoxic and ischemic nature of human injury more closely than the contusion SCI model (Zhang et al., 1993). At 1 day post-injury in the rat, 44% of neurons, assessed by NeuN immunostaining, were lost from the injury epicenter (Huang et al., 2007). This loss of neurons increased to 73% by 3 days and 81% by 1 month post-injury.

Strong correlations between neurological function and the extent of white matter damage following experimental SCI (Noble and Wrathall, 1989; Fehlings and Tator, 1995; Basso et al., 1996b; Gruner et al., 1996) have led to an increased interest in the axonal tracts, and their dysfunction is now understood to play a key role in neurological outcome following both experimental and human SCI (Eidelberg et al., 1977; Blight, 1991; Nashmi and Fehlings, 2001b; You et al., 2003; Norenberg et al., 2004). Compression SCI results in axonal dysfunction, as demonstrated by the accumulation of β-amyloid precursor protein (β-APP) (Li et al., 1995) as well as the loss of cytoskeletal proteins (microtubule associated protein-2, MAP-2) and demyelination at the injury epicenter (Holtz et al., 1990; Yu et al., 2000). However, a characterization of these alterations away from the lesion site, in the regions that are likely to be affected by secondary injury, has not been performed in this injury model. In addition phosphorylated and

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Abbreviations: β-APP, β-amyloid precursor protein; CST, corticospinal tract; DC, dorsal column; DLWM, dorsolateral white matter; FITC, fluorescein isothiocyanate; IR, immunoreactivity; MAP-2, microtubule associated protein-2; MBP, myelin basic protein; NF-200, neurofilament-200; PB, phosphate buffer; PBS, phosphate-buffered saline; PFA, paraformaldehyde; PO, propylene oxide; SCI, spinal cord injury; TRITC, tetramethylrhodamine isothiocyanate; VLWM, ventrolateral white matter; VWM, ventral white matter.

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non-phosphorylated neurofilament epitopes, which have been reported to be affected differently depending on the type of SCI (Choo et al., 2008), have not been investigated in a static compression model. Therefore, in the present study, we use immunohistochemistry with a range of markers, and electron microscopy to examine the spatiotemporal development of white matter pathology following injury in a rat static compression model of SCI.

EXPERIMENTAL PROCEDURES

Animal care and surgical procedures

All animal procedures were approved by the Animal Care Committee of Queen Mary University of London and the United Kingdom Home Office in accordance to the UK Animals (Scientific Procedures) Act of 1986. The spinal cord was injured at vertebral thoracic level 12 (T12) using a previously established static compression model (Nystrom et al., 1988; Huang et al., 2007). Adult female Sprague–Dawley rats (230–255 g) were anesthetized using 2–3% halothane (Meril, UK) and the spinal cord at the T12 vertebral level was exposed by laminectomy with the dura intact. The T11 and T13 spinal processes were clamped to prevent movement of the spinal cord and compression was applied by suspending the base of the compression platform (area 2 × 5 mm) onto the exposed spinal cord under microscopic control. A weight of 50 g was then applied statically to the platform for 5 min. Muscle layers were then sutured and skin layers closed with wound clips. Following surgery, rats were given buprenorphine intramuscularly (0.12 mg/kg, Reckitt Benckiser, UK). Manual bladder expression was performed twice a day for the first week and once daily thereafter, for the rats that were kept past 7 days post-injury, until the establishment of reflex voiding (normally within 2 weeks post surgery). At various post-surgery intervals (1, 3, 7 days, and 6 weeks post-injury), rats were deeply anesthetized with sodium pentobarbital (60 mg/kg, Sagatal, France) and perfused for tissue processing. Uninjured rats, either naïve rats or rats whose spinal cord had been exposed by laminectomy at T12, were used as controls.

Tissue processing

Rats were perfused via the ascending aorta with 0.01 M phosphate-buffered saline (PBS), followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB), pH 7.4. The 5-mm segment of the spinal cord containing the compression injury site, and the adjacent rostral and caudal 5-mm segments, were dissected out and post-fixed in 4% PFA for 2 h at room temperature and then cryoprotected in 30% sucrose in 0.1 M PB overnight at 4 °C. All segments were embedded in OCT and stored at −80 °C until required.

Immunohistochemical analysis

Serial 10-μm transverse cryostat sections of the lesion epicenter were collected and blocked in 10% normal donkey serum for 1 h. Sections were processed for immunohistochemistry using the following primary antisera: rabbit β-APP (1:500; Zymed, San Francisco, CA, USA) to examine axonal transport dysfunction, mouse SMI31 and SMI32 (both 1:1000; Sternberger Monoclonals Inc., Lutherville, MA, USA) to label phosphorylated and non-phosphorylated 200 kD neurofilament, respectively, rabbit neurofilament-200 (NF-200) to label both phosphorylated and non-phosphorylated 200 kD neurofilament, mouse MAP-2 (1:1000; Sigma, UK) to label cell bodies and their dendrites and mouse myelin basic protein (MBP) (1:500; Roche, UK) to label central nervous system (CNS) myelin. Following a 48-h incubation with the primary antibody, sections were washed in PBS and incubated for 2 h in the appropriate secondary antibody conjugated to either fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC) (both at 1:600; Jackson Immunoresearch Laboratories, USA) before being counterstained with the fluorescent nuclear dye bisbenzimide (Hoechst 33342, 2 μg/ml; Sigma, UK) and coverslipped in PBS glycerol. Prior to incubation with the MBP antibody, sections were delipidated by dehydrating for 1 min in each of the following ethanol solutions; 50%, 70%, 90%, 95%, 2 × 100%. Sections were then incubated in chloroform for 5 min, rehydrated through the same ethanol solutions in reverse order and dipped in distilled water, before being washed in PBS and blocked with 10% normal serum as before. This delipidation process enhanced the specificity of the myelin labeling without altering the labeling of other antibodies. To achieve even SMI32 labeling, sections were post-fixed in ice-cold methanol for 5 min and then washed four times for 5 min in PBS prior to blocking with 10% normal serum.

Image and data analysis

Sections were viewed on a Leica epifluorescence microscope (Wetzlar, Germany) using L4 (FITC), Y3 (TRITC) or DAPI (4′,6-diamidino-2-phenylindole) filter blocks. Images were taken using a Hamamatsu C4742-95 digital camera (Herrsching, Germany) and HiPic software (Hamamatsu, UK). Figures were prepared using Adobe Photoshop (Adobe Systems, San Jose, CA, USA).

Three sections were analyzed for each rat, each anatomical level (rostral, epicenter, and caudal segments), each anatomical region (see below), and each antigen. β-APP labeled axons were counted by a blinded observer using a 40× objective in four specified regions (size 340 μm × 272 μm = 92,691 μm^2) of the white matter, namely the dorsal column (DC), corticospinal tract (CST), ventral white matter (VWM) and ventrolateral white matter (VLWM, see Fig. 1A). MBP staining was quantified by counting MBP immunoreactive myelin rings in a 100 μm × 100 μm area of the same four white matter regions. The number of SMI31 and SMI32 labeled axons was determined in the DC, VWM and VLWM using QWin software (Leica, UK). One image per region of interest, taken at 40× magnification, was converted into a binary image of the labeling by segmenting based on gray level. Ten frames