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Effect of stab injury in the rat cerebral cortex on temporal pattern of expression of neuronal cytoskeletal proteins: An immunohistochemical study

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ABSTRACT

Compelling evidence now points to the critical role of the cytoskeleton in neurodegeneration. In the present study, using an immunohistochemical approach, we have shown that cortical stab injury (CSI) in adult Wistar rats significantly affects temporal pattern of expression of neurofilament proteins (NFs), a major cytoskeleton components of neurons, and microtubule-associated proteins (MAP2). At 3 days post-injury (dpi) most of the NFs immunoreactivity was found in pyknotic neurons and in fragmentized axonal processes in the perilesioned cortex. These cytoskeletal alterations became more pronounced by 10 dpi. At the subcellular level CSI also showed significant impact on NFs and MAP-2 expression. Thus, at 3 dpi most of the dendrites disappeared, while large neuronal somata appeared like open circles pointing to membrane disintegration. Conversely, at 10 dpi neuronal perikarya and a few new apical dendrites were strongly labeled. Since aberrant NF phosphorylation is a pathological hallmark of many human neurodegenerative disorders, as well as is found after stressor stimuli, the present results shed light into the expression of neurofilaments after the stab brain injury.

Introduction

Traumatic brain injury (TBI) is associated with neuropathological changes. These changes evolve over a period of minutes to months after TBI leading to degeneration of primarily unaffected neurons via secondary axotomy as the result of progressive structural damage in neurons (Gennarelli, 1996; Maxwell et al., 1997; Povlishock and Katz, 2005). Delayed axotomy is believed to occur subsequently to initial changes in permeability of the axonal membrane and disruption of certain elements of cytoskeleton, particularly axonal neurofilaments (Pettus et al., 1994). Thus, the consequence of neuronal degeneration is an accumulation of various proteins including neurofilament proteins, as major cytoskeleton components of neuronal cells, and microtubule-associated proteins (MAPs) (Caner et al., 2004). MAP2 is a member of the MAPs family, with a particular role in stabilization of microtubules by crosslinking them with neurofilaments (NFs) and other microtubules, and in regulation of inter-microtubular spacing (Chapin and Bulinski, 1992). Integration of NFs and MAP-2 is essential for normal functioning of nerve cells, especially for the performance of axonal and dendritic transport (Caner et al., 2004).

Neurofilaments (NFs) are intermediate filaments of the neuronal cytoskeleton that provide the mechanical stability of the cells and have a fundamental role in axonal transport (Liu et al., 2004; Barry et al., 2007). While NFs are present in dendrites and perikarya, they are abundantly present in axons, maintaining the structural integrity and caliber of axons through influencing the conduction velocity of nerve impulses (Shea and Lee, 2013). According to their molecular size, neurofilament subunits are divided into three groups: light (NF-L, 68 kDa), medium (NF-M, 150 kDa) and heavy (NF-H, 200 kDa) (Nixon and Shea, 1992), along with α-internexin (Int) (Yuan et al., 2012). NF-H plays an essential role in axonal outgrowth and stabilization (Lee and Shea, 2014), while NF-M is involved in stabilization of the filament network and helps in longitudinal extension (Elder et al., 1998; Barry et al., 2012), whereas NF-L is essential for the correct assembly of neurofilaments (Yuan et al., 2012). All NFs share a highly conserved central rod domain, which is responsible for the formation of

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coiled-coil structures (Herrmann et al., 2009). The NF-M and NF-H carboxy-terminal regions contain multiple repeats of the motif Lys-Ser-Pro (KSP), which represent a site for phosphorylation (Lariviere and Julien, 2004; Barry et al., 2010). Recent studies imply that phospho-mediated interactions of NF-H and NF-M are regulated by a complex cascade of kinase and phosphatase activity (Lee et al., 2014). Further, NF-M have a critical role in myelination by regulating axonal diameter, by a mechanism that is independent of KSP phosphorylation (Barry et al., 2012). A growing body of evidence indicates that NFs are changed in different neurodegenerative diseases, probably through aberrant signal transduction leading to altered phosphorylation of NFs, disruption of NFs transport and their massive accumulation (Perrot et al., 2008; Wang et al., 2012). Accumulation of NFs is found in amyotrophic lateral sclerosis, Alzheimer's disease, Parkinson disease, Charcot–Marie disease, diabetes neuropathy and giant axonal neuropathy (Al-Chalabi and Miller, 2003; Wang et al., 2012; Yuan et al., 2012).

NF protein expression is closely linked with continuous growth of normal axons and impaired axonal regeneration (Wang et al., 2012). In addition, phosphorylated NF is responsible for regulating a variety of biological functions such as axonal transport in neurons (Lee et al., 2011a,b). However, the specificity and degree of remodeling that can occur in response to CNS injury has not been completely elucidated. It was shown that cortical neurons demonstrated axonal and synaptic remodeling in response to various models of TBI (King et al., 2001; Majewska et al., 2006). In this paper, using immunohistochemistry, we have investigated the temporal expression profiles of cytoskeletal proteins NF-H, NF-M, NF-L and MAP2 after unilateral cortical stab injury (CSI) in adult rats.

Materials and methods

Animals

The experiments were performed on adult male Wistar rats (10 weeks old) obtained from the vivarium of the Institute for Biological Research "Sinisa Stankovic" (Belgrade, Serbia). At the beginning of the experiments there was no statistically significant difference in body weight (250 ± 30 g), within the group as well as between the groups. All animals were housed four per cage, under standard conditions (23 ± 2 °C, 50–60% relative humidity, 12 h/12 h light/dark cycle, with free access to food and water). Before the surgery animals were randomly divided into the following groups: control group (C) of intact rats ($n = 3$); sham-operated (S) group of animals that underwent surgical procedure without skull opening ($n = 5$), and lesion group (L) with animals subjected to cortical stab injury (CSI) ($n = 5$). Animals were sacrificed by decapitation under deep anesthesia at 3 and 10 days post-injury (dpi). All experimental procedures were in compliance with the EEC Directive (86/609/EEC) on the protection of animals used for experimental and other scientific purposes, and were approved by the Ethical Committee for the Use of Laboratory Animals of the Institute for Biological Research "Sinisa Stankovic", University of Belgrade. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Cortical stub injury model

Before starting the surgical procedure, rats were weighed and anesthetized with ether. After the onset of anesthesia, rats were placed in a stereotaxic frame and heads were shaved. The head was incised using a scalpel blade. A handheld 1-mm diameter dental drill was inserted vertically in the cranium on the left side. The coordinates of stab lesion to the left cortex were as follows: 2 mm posterior to the bregma, 2 mm lateral from the midline, and to a depth of 2 mm into the brain (Paxinos and Watson, 2005). Rats from the sham control groups underwent anesthesia, scalp incision along the midline and closure. Intact animals were untreated age-matched controls. After the surgery, the rats were kept warm and left to recover.

Histopathology

Histological changes were assessed in the brain sections obtained at the level of the created lesion. After decapitation, brains were quickly removed and fixed in 4% paraformaldehyde for 12 h. For cryoprotection tissue was transferred into graded sucrose. Brains were frozen in 2-methyl butane and kept at −80 °C until sectioning on a cryotome. The brains were cut in coronal sections 25 μm thick and mounted on Superfrost® glass slides, dried for 2 h at room temperature and stored at −20 °C until staining.

Immunohistochemistry

Frozen coronal 25 μm thick sections were processed for immunohistochemistry to detect histological and pathological changes. Immunohistological data were determined on 5–7 slides per rats. Since no difference in the pattern of NFs immunostaining between the intact and sham-operated controls was seen (data not shown), all comparisons were done with the intact control. Labeling for NF-H, NF-M and NF-L was performed according to the standard procedure. Briefly, after washing in phosphate buffered saline (PBS), blocking was done using 5% normal donkey serum (Sigma–Aldrich, Germany). Sections were incubated overnight at 4 °C with primary antibodies: mouse anti-NF-H (1:500; Millipore, Germany); rabbit anti-NF-M (1:250; Millipore, Germany); rabbit anti-NF-L (1:250; Millipore, Germany). Using appropriate peroxidase linked secondary antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA, USA), the immunoreaction products were visualized with 3′3-diaminobenzidine (DAB; Dako, Glostrup, Denmark) according to manufacturer's instructions. The specificity of the staining was tested on the sections treated in the same way, but with the omission of the primary antibodies. The sections were examined and photographed with Carl Zeiss Axiovert microscope (Zeiss, Gottingen, Germany). Images were acquired using 10×, 20× and 40× objectives.

Immunofluorescence

Normal donkey serum (Sigma–Aldrich) was used for blocking of non-specific labeling as 5% solution in PBS. Colocalization of NF-M and NF-L with marker of dendrites MAP-2 was examined with double immunofluorescence labeling using rabbit anti-NF-M (1:250; Millipore, Germany); rabbit anti-NF-L (1:250; Millipore, Germany) and mouse anti-MAP-2 (1:100; Sigma–Aldrich, Germany), respectively. Immune complexes were visualized with donkey anti-rabbit IgG Alexa Fluor 555 (1:200; Invitrogen, Carlsbad, CA, USA), or with donkey anti-rabbit IgG Alexa Fluor 488 and donkey anti-mouse Alexa Fluor 555 (1:200; Invitrogen, Carlsbad, CA, USA). For the negative control, sections were incubated with appropriate secondary antibodies without the primary antibody. The sections were mounted in Mowiol (Calbiochem, San Diego, CA) and examined under a Carl Zeiss Axiovert fluorescence microscope (Zeiss, Gottingen, Germany) equipped with a camera and EC Plan-Apochromat 100× objective, using the ApoTome software module for generating optical sections through fluorescence samples. All images were acquired with the 100× objective.