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Proteome analysis of lumbar spinal cord from rats submitted to peripheral lesion during neonatal period

Erich Castro-Dias · André S. Vieira · Claudio C. Werneck · Francesco Langone · José C. Novello · Daniel Martins-de-Souza

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Abstract Every year traumatic peripheral nerve injuries (TPNI) result in considerable physical disability across the world; the mechanisms of plasticity and reorganization of spinal cord circuits following such injuries are complex and not completely understood. A comparative proteome analysis between neonatal rats submitted to peripheral lesion and controls was performed; a number of differentially expressed proteins involved in oxidative stress response, energy metabolism and cytoskeleton rearrangement were revealed, which may support future studies to help in the understanding and a posteriori the treatment of TPNI.

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E. Castro-Dias · C. C. Werneck · J. C. Novello · D. Martins-de-Souza
Laboratório de Proteômica, Depto de Bioquímica, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Rua Monteiro Lobato, s/n, CP 6109, Campinas, SP 13.083-970, Brazil

A. S. Vieira · F. Langone
Depto de Genética e Evolução e Bioagentes, Instituto de Biologia, Universidade Estadual de Campinas (UNICAMP), Rua Monteiro Lobato, s/n, CP 6109, Campinas, SP CEP 13.083-970, Brazil

D. Martins-de-Souza (✉)
Max Planck Institute for Psychiatry, Kraepelinstraße 2-10, 80804 Munich, Germany
e-mail: danms90@gmail.com

D. Martins-de-Souza
Laboratório de Neurociências (LIM-27), Inst. Psiquiatria, Fac. de Medicina da Universidade de São Paulo, Rua. Dr. Ovidio Pires de Campos, 785, São Paulo, SP 05403-010, Brazil

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Introduction

Every year, traumatic peripheral nerve injuries (TPNI) result in considerable physical disability across the world owing to motor vehicle or industrial accidents, lacerations by glass or knife or fan, ischemia, long bone fractures and also thermal and electric shock (Robinson 2000; Campbell 2008). Such lesions lead to deep modification and reorganization of both, peripheral nervous systems, which attempts to fit the anomaly through the reinnervation of the affected tissue by intact axons, and central nervous system (CNS) specifically through the spinal cord (Zochodne and Levy 2005).

The mechanisms of plasticity and reorganization of spinal cord circuits following TPNI are complex, leading either to adaptative benefits that can drive possibly regeneration or collateral effects such as central or peripheral hyperexcitability and pain. Such mechanisms are not completely understood and are essential due to their clinical relevance in the development of corrective treatments and surgeries for neurotrauma.

Neonatal rats have been the most employed animal models to study TPNI mechanisms since the spinal cord in the post-natal period shows intense neuroplasticity (Hensch 2005; Ohno and Sakurai 2005). The post-natal period represents the interval which external (like an injury) and internal stimuli has the ability to influence the system development. As the knowledge of degenerative mechanisms is very deficient and could be an interesting approach to study TPNI and their direct consequences, peripheral nerve injuries in neonates are used as a model in order to

elucidate the transduction pathways and protein expression which leads nerve cells to death. The proteome analysis of such models may be used to this purpose, since proteomics has revealed a world of answers through the analyses of differentially expressed proteins comparing two or more biological states (Martins-de-Souza 2009).

Here we present our findings in the comparative proteome analysis of the spinal cord from injured neonates and healthy controls using the traditional combination of two-dimensional gel electrophoresis (2-DE) for protein separation and mass spectrometry (MS) for protein identification. Such analysis allows revealing differentially expressed proteins that can lead to a better comprehension of the biochemical mechanisms post-TPNI as well as potential biomarker candidates (PBC, Martins-de-Souza 2010) to spinal cord lesion.

Materials and methods

Two-day-old Wistar rats, along with their mothers, were obtained from the State University of Campinas Multidisciplinary Center for Biological Investigation in Laboratory Animal Science (CEMIB). Rats were housed in plastic cages in a ventilated rack (Alesco model 9902.001; Alesco, Campinas, SP, Brazil) with a 12 h light–dark cycle at 22 ± 1°C. For sciatic nerve lesion, 2-day-old male rats were anesthetized by hypothermia through immersion in crushed ice for 3–5 min. Both the left and the right sciatic nerves were then cut at the mid-thigh level. Approximately 3 mm of the distal stumps was removed and the skin was sutured (Silk 8-0, Ethicon, USA). The rats were allowed to recover from the anesthesia under an incandescent lamp and were returned to their mothers. Unlesioned male rats were used as controls. As a control for sciatic nerve transection efficiency, a histological analysis was performed in the spinal cord of one animal from each group. Only injured animals displayed a bilateral degeneration of spinal motoneurons (Supplementary Fig. 1).

All experiments were conducted in accordance with the principles and procedures described by the NIH Guidelines for the Care and Use of Experimental Animals and were approved by the State University of Campinas Committee of Animal Care (Protocols CEEA-UNICAMP 509-1 and 878-2).

For protein extractions, rats were anesthetized by hypothermia on the 5th day post-axotomy, and killed by decapitation. The lumbar spinal cord was rapidly removed, immediately frozen in liquid nitrogen, and stored at −80°C. The lumbar spinal cords of control rats with matching age were collected and stored as described above.

The nine injured and the nine unlesioned rats had their proteins from the spinal cord extracted and quantified according to Martins-de-Souza et al. (2007). The 2-DE gels of two distinct pH ranges [3 to 10 non-linear (3–10NL) and 4 to 7 linear (4–7L)] were carried out in triplicate using three distinct pools of three injured and three healthy controls. Proteome separation, quantitation of differences in protein expression (±1.8-fold difference (Mann–Whitney test) considering the limits of 2-DE post-stained methods Martins-de-Souza et al. 2008), MS protein identification and Western blot (WB) analysis were performed as previously described in detail in Martins-de-Souza et al. (2009a). All experiments were performed in triplicate. Proteins that appeared to be differentially expressed in two out of three experiments were considered as differentially expressed.

Results

The proteomes of injured and unlesioned animals were analyzed by 2-DE revealing 627 and 424 protein spots (on average) in 3–10NL and 4–7L 2-DE profiles, respectively (Supplementary Fig. 2). The 2-DE gels from injured and unlesioned animals were computationally matched and compared revealing six differentially expressed spots statistically significant (Mann–Whitney; $p < 0.05$) in 3–10NL gels and five differentially expressed spots statistically significant in 4–7L gels (Table 1), summing eleven differentially expressed spots. Since spots 1, 5, and 6 from 3–10NL gels were not sensible enough to be identified by MS, we identified eight differentially expressed proteins in total.

Discussion

Glutathione S-transferase Mu 1 (Gstm1) is a multifunctional enzyme that plays roles in cellular detoxification, reduces glutathione (Hayes et al. 2005) and metabolizes reactive oxygen species (ROS) avoiding oxidative stress (Oakley 2005). After nerve injury, which occurs during a neurodegenerative course, inflammatory processes are triggered, increasing ROS (Floyd 1999). As a response, there is an overexpression of enzymes responsible for combating oxidative stress, such as Gstm1, which we found upregulated in injured animals. Nevertheless, superoxide anion, a kind of ROS, is responsible for damaging the structure of the cellular and mitochondrial lipid layer, generating mutagenic compounds. Previous reports have shown that Gstm1 is capable of conjugating those compounds with glutathione, neutralizing their hazardous effect (Hayes et al. 2005). Given the importance of Gstm1 regarding inflammatory and oxidative stress response, which occurs in injured animals, we performed a validation analysis of this enzyme by WB in a distinct set of injured

Table 1 Differentially expressed proteins ($p < 0.05$) found when injured rat samples were compared to controls

2DE Gel	Spot number	Protein	Gene name	Swiss-Prot access	p*I*	MW	Peptide hits	Coverage (%)	*p* value	Reg. injured	Fold difference
3–10	2	Glutathione S-transferase Mu 1	Gstm1	P04905	8.4	25	12	62	7.3E-08	↑	2.5
3–10	3	Catalase	Cat	P04762	7.1	59	16	42	2.6E-08	↑	2.6
3–10	4	Glucose-6-phosphate 1-dehydrogenase	G6pd	P05370	6.2	59	20	51	3.7E-05	↑	1.9
4–7	1	Annexin A6	Anxa6	P48037	5.4	76	11	21	4.7E-06	↑	1.8
4–7	2	Protein disulfide isomerase A3	Pdia3	P11598	5.8	54	19	34	1.9E-07	↑	2.1
4–7	3	Protein disulfide isomerase A3	Pdia3	P11598	5.8	54	11	22	5.1E-07	↓	1.9
4–7	4	Ubiquitin carboxyl-terminal hydrolase	UchL1	Q00981	5.1	25	7	35	0.0001254	↓	1.8
4–7	5	Tubulin beta-5 chain	Tubb5	P69897	4.8	50	10	23	2.3E-11	↑	2.9

and control animals. The upregulation of Gstm1 was validated as shown in Fig. 1, confirming our findings.

Catalase (Cat), likewise as Gstm1, fights cellular oxidative stress by transforming hydrogen peroxide (H_2O_2) in H_2O and O_2, detoxifying tissues and cells under oxidative stress as occurs during degenerative processes (Dringen et al. 2005). Cat was found in the present study upregulated in injured animals as well as Gstm1. Neonates who suffered nerve injury are under degenerative conditions and apoptotic events, bringing as a consequence increased rates of ROS and its secondary metabolites (Singh et al. 2009).

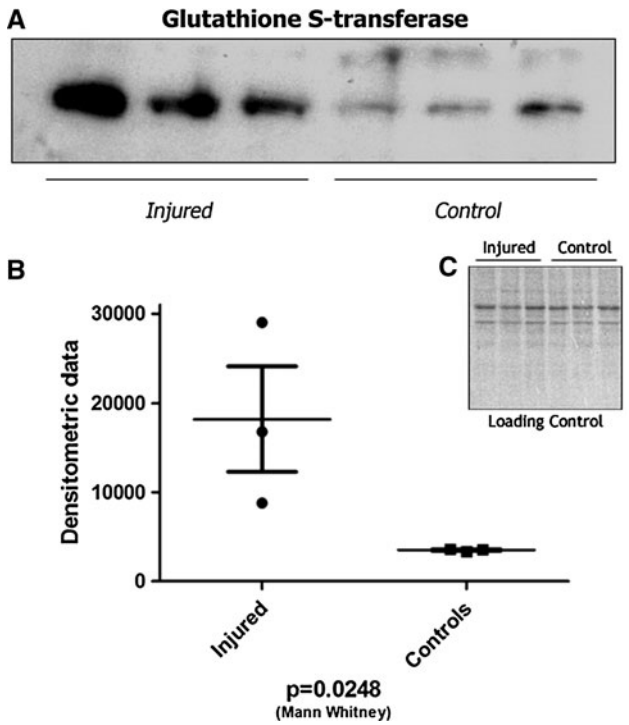

Fig. 1 The validation of Gstm1, a potential biomarker candidate for spinal cord lesion. **a** Gstm1 WB profile in injured and control samples. **b** The densitometric data from Gstm1 bands. **c** Ponceau-stained WB loading control

Among many functions, Protein disulfide isomerase A3 (Pdia3) plays roles in protein folding, cell redox homeostasis and apoptosis regulation. The excess of ROS, such as nitric oxide, may trigger the upregulation of Pdia3 since this protein has regulatory function in cellular oxidation–reduction reactions (Kim et al. 2009). It is interesting to note that Pdia3 was identified in two different spots in the 2-DE gels (spots 2 and 3 of the 4–7 2-DE gel). Spot 2 is upregulated in injured rats and spot 3 upregulated in controls, which means that this protein would be upregulated in both conditions in 2 different spots. However, the p*I* shift between spots 2 and 3 means that a post-translational modification is occurring, that may be necessary to the Pdia3 functioning. This is an interesting feature that 2-DE gels may reveal, meaning that the different states of the same protein may be differentially expressed under different conditions. Pdia3 is qualitatively, not quantitatively, differentially expressed.

Oxidative stress conditions as described above may bring consequences to energy metabolism processes, since the mitochondrial membranes may be damaged by ROS. In injured rats, we observed an upregulation of glucose-6-phosphate dehydrogenase (G6pd), an enzyme of the pentose phosphate pathway (PPP). The PPP's main function is the generation of redox potential through the production of NADPH, strictly necessary for oxidative phosphorylation, which occurs in the mitochondrial membrane. Assuming that oxidative stress conditions may damage the mitochondrial membrane, the G6pd upregulation might be a natural response to the imbalanced energy production in injured rats. Moreover, NADPH plays an important role in cell defense against ROS because of its role as an electron donator. Higher amounts of ROS in injured rats trigger a cellular response against oxidative stress such as the higher production of NADPH.

It is known that under stressful or situations of illness, cells suffer cytoskeleton rearrangements, which are a natural reflex of the differential expression of cytoskeleton proteins. Tubulin beta-5 chain (Tubb5) was found upregulated in injured animals. Tubb5 may be involved in the cellular

reconstruction in response to the peripheral lesion (Fournier and McKerracher 1995). The upregulation of tubulin subunits has been previously observed in injured animals (Hoffman et al. 1993; Kang et al. 2006), supporting our data.

Annexin A6 (Anxa6) that was found upregulated in injured animals is widely present in both sensitive and motor neurons (Naciff et al. 1996). Anxa6 binds negatively charged phospholipids (Thomas et al. 2002), which might be helpful in cellular reconstruction. Moreover, the nerve injury caused cell disruption and consequently a Ca^{+2} overflow in the spinal cord tissue. Anxa6 is directly involved in Ca^{+2} binding and transport, which can explain its upregulation, corroborating the previous findings in injured spinal cord analysis (Kang et al. 2006). Ca^{+2} channels were reported as important for axon membrane regeneration and repair following a nerve injury (Kulbatski et al. 2004; Nehrt et al. 2007). It is important to comment that apoptotic processes, which must be occurring in injured animals, are Ca^{+2}-dependent and the differential expression of Anxa6 might be related to such processes.

The main function of Ubiquitin carboxyl-terminal hydrolase (UchL1 downregulated in injured rats) is the processing of ubiquitin precursors. Ubiquitins, which play a pivotal role in protein degradation, synaptic function and neuronal apoptosis (Hegde and DiAntonio 2002), have been also involved in axonal loss and cell death (Mukoyama et al. 1989), schizophrenia (Martins-de-Souza et al. 2009b) and neurodegenerative disorders such as Parkinson and Alzheimer (Choi et al. 2004). The involvement of UchL1 in neurodegeneration added to the upregulation of Anxa6 and its involvement to Ca^{+2} and the oxidative stress event, proved by the upregulation of Gstm1, Cat and Pdia3, corroborates and better explains the injury mechanisms. Moreover, oxidative stress may impair UchL1 functioning (Choi et al. 2004).

Conclusions

Our data support not only the oxidative stress event caused by the injury but also the involvement of energy metabolism and cytoskeleton rearrangement. Such results are in line with the degenerative process that is occurring in injured animals. This study may trigger the importance of protein expression studies in TPNI, particularly aiming for a better comprehension of the biochemical mechanisms post-TPNI and also potential protein marker candidates, which may help towards more effective treatments in future.

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