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Proteomic analysis of opossum *Monodelphis domestica* spinal cord reveals the changes of proteins related to neurodegenerative diseases during developmental period when neuroregeneration stops being possible

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ABSTRACT

One of the major challenges of modern neurobiology concerns the inability of the adult mammalian central nervous system (CNS) to regenerate and repair itself after injury. It is still unclear why the ability to regenerate CNS is lost during evolution and development and why it becomes very limited in adult mammals. A convenient model to study cellular and molecular basis of this loss is neonatal opossum (*Monodelphis domestica*). Opossums are marsupials that are born very immature with the unique possibility to successfully regenerate postnatal spinal cord after injury in the first two weeks of their life, after which this ability abruptly stops. Using comparative proteomic approach we identified the proteins that are differentially distributed in opossum spinal tissue that can and cannot regenerate after injury, among which stand out the proteins related to neurodegenerative diseases (NDD), such as Huntington, Parkinson and Alzheimer's disease, previously detected by comparative transcriptomics on the analog tissue. The different distribution of the selected proteins detected by comparative proteomics was further confirmed by Western blot (WB), and the changes in the expression of related genes were analysed by quantitative reverse transcription PCR (qRT-PCR). Furthermore, we explored the cellular localization of the selected proteins using immunofluorescent microscopy.

To our knowledge, this is the first report on proteins differentially present in developing, non-injured mammalian spinal cord tissue with different regenerative capacities. The results of this study indicate that the proteins known to have an important role in the pathophysiology of neurodegeneration in aged CNS, could also have an important physiological role during CNS postnatal development and in neuro-regeneration process.

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1. Introduction

The inability of the adult mammalian CNS to regenerate after injury is one of the greatest challenges of modern neurobiology. Even though our understanding of molecular and cellular mechanisms that promote or inhibit neuronal regeneration is expanding [1,2], it is still unclear what are the key differences between the neuronal systems that can and cannot regenerate, and how they can be manipulated to revert the outcome. For reasons yet unknown, with development and age, mammalian CNS loses the

capacity for functionally relevant repair after injury [3,4], as it is observed on the evolutionary scale. Although the CNS is more plastic early after birth than in the adult, the postnatal mammalian CNS usually displays limited regenerative capacities and the precise cellular and molecular basis for this developmental loss are mostly unclear. Marsupials (non-placental mammals) are born very immature and thus unique in their possibility to successfully regenerate postnatal CNS after injury. In short-tailed gray opossums *Monodelphis domestica*, new born animals correspond roughly to 12-day mouse or rat embryos [5], and they have the ability to fully functionally regenerate cervical spinal cord until the postnatal day 12, while in the less mature lumbar segments regeneration continues until postnatal day 17, approximately [6]. Thus, the opossums represent the unique opportunity to achieve

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and study mammalian CNS that can regenerate without the need of intrauterine surgery of pregnant mothers. In the previous studies [7,8], differentially expressed genes were identified in opossum spinal cords during the critical period of development when regeneration stops being possible, revealing the molecules involved in nucleic acid management, protein synthesis and processing, control of cell growth, structure and motility, cell signaling, extracellular matrix molecules and their receptors. The bottleneck of the genomic approach is the difficulty to select the relevant molecules from the comprehensive lists of the candidates. Thus, the previous data were now upgraded with the proteomic research, to select the overlapping molecules as the most promising candidates controlling regeneration, to be tested in functional studies.

Our study is the first attempt to compare the proteomes of non-injured mammalian CNS tissue with different regeneration capacities. This approach was chosen to reveal the physiological properties of neuronal tissue related to regeneration, differently to many other studies where the post-injury protein changes were studied, detecting the molecules involved in the pathophysiology of spinal cord injury (SCI), i.e. related to neuroinflammation, excitotoxicity and oxidative stress [9–12].

The results of this study indicate that proteins related to neurodegeneration in aged CNS, could have an important physiological role during CNS postnatal development, particularly that they could be involved in molecular and cellular mechanisms underlying neuroregeneration [13]. Further studies are necessary to clarify if the identified molecules are actually important for neuroregeneration/neurodegeneration processes and if therefore they could be possible therapeutic targets for treatment of CNS injuries.

2. Materials and methods

2.1. Ethics statements and animals

In this work, South American gray short-tailed opossum (*Monodelphis domestica*) pups of both sexes, at postnatal day (P) 5, 18 and 30, were used. The colony was maintained at the animal house facility of the University of Trieste, in accordance with the guidelines of the Italian Animal Welfare Act, and their use was approved by the Local Veterinary Service, the Ethics Committee board and the National Ministry of Health (Permit Number: 1FF80.N.9Q3), in accordance with the European Union guidelines for animal care (d.1.116/92; 86/609/C.E.). The animals are housed in standard laboratory cages in a temperature and humidity controlled environment (27–28 °C; 50–60% humidity) with a 12/12 h light/dark cycle and *ad libitum* access to food and water. All efforts were aimed at reducing the number of animals used and at minimizing their suffering.

2.2. Spinal cord preparation

The whole spinal cord tissue was lysed with RIPA lysis buffer (Thermo Fisher Scientific, USA) supplemented with protease and phosphatase inhibitors (Roche Applied Science, CH). Tissue was then homogenized with an electric homogenizer and sonicated 3 × 5 s on ice. Finally, the suspension was centrifuged for 30 min at 14,000 rpm at 4 °C. Supernatants of whole cell lysate were used for further analysis or stored at –80 °C.

2.3. Mass spectrometry (MS)

Nano-HPLC-MS/MS was used to analyze the protein gel bands. Excised protein bands were digested in-gel using trypsin. LC-MS/MS analysis was done at the Proteome Center Tübingen on an Easy-nLC 1200 (Thermo Scientific, USA) coupled to a QExactive HF

mass spectrometer (Thermo Scientific, USA). The 7 most intense precursor ions were sequentially fragmented in each scan cycle using HCD fragmentation. MS data were processed using the MaxQuant software suite v.1.5.2.28. Database search was performed using the Andromeda search engine. Spectra were searched against an Uniprot *Monodelphis domestica* database, as well as a database containing 248 commonly observed contaminants. In database search, semi-specificity was required for trypsin and up to two missed cleavages were allowed. Carbamidomethylation of cysteine was set as fixed modification, protein N-terminal acetylation, and oxidation of methionine were set as variable modifications. False Discovery Rate (FDR) was set to 1% on protein as well as peptide level. A minimum of two unmodified peptide counts were required for the respective protein quantification. The label-free algorithm was enabled, as was the “match between runs” option.

2.4. Functional categorization of the detected proteins

To obtain an overview of the biological significance of the proteins detected by MS, the candidates were categorized according to their main biological functions collected from UniProt protein knowledge database (<http://www.uniprot.org>) and PubMed (<http://www.ncbi.nlm.nih.gov>). Functional categorization was done using PANTHER classification system (<http://pantherdb.org/>).

2.5. Western blot

Protein concentration in samples was determined using Qubit™ protein kit (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. Thirty micrograms of proteins were resolved by SDS-PAGE and transferred to nitrocellulose membrane. After blocking in 4% non-fat milk, membranes were probed with primary antibodies raised against GFAP (Sigma-Aldrich, G3893, 1:1000), PAX6 (Abcam, ab5790, 1:1000), PAX2 (Abcam, ab79389, 1:1000), GAP43 (Abcam, ab75810, 1:20,000), MBP (Santa Cruz, sc-13912, 1:500) and BLBP (Abcam, ab32423, 1:1000) overnight at 4 °C. After washing in Tris-buffered saline (TBS) with Tween 20, membranes were incubated with secondary antibody 1 h at room temperature (RT). The signal was visualized with LiteAblot turbo chemiluminescent substrate (Euro-Clone, Italy) and the densitometric analysis was performed by ImageJ software (NIH, Bethesda, Maryland, USA). To normalize the signal, membranes were incubated with HRP conjugated GAPDH (ProteinTech, HRP-60004, 1:10,000).

2.6. RNA extraction and qRT-PCR

Total RNA was isolated using a Monarch Total RNA Miniprep kit (NEB, USA) according to the manufacturer's instructions. RNA concentration and quality was measured using UV/VIS spectrophotometer (BioDrop ILITE, UK). RNA was transcribed using the ProtoScript® First Strand cDNA Synthesis Kit (NEB, USA) according to the manufacturer's instructions. RT-PCR was performed with SYBR green I Master Mix using LightCycler 480 (Roche Holding AG, CH). The relative mRNA expression levels of all genes of interest (GOI) were normalized to the level of the reference gene (GAPDH) and calculated by equation $2^{-(Ct \text{ value for GOI} - Ct \text{ value for GAPDH})}$. The primers used were: GAPDH - forward 5'-ATGCCCAATGTTCTGTATG-3', reverse 5'-GTCATGAGTCCTCCCACTGC-3'; GFAP - forward 5'-AACAAAACAAGGTCCTGGCA-3', reverse 5'-GTGGTCAGATCTTCAGCCAG-3'; PAX6 - forward 5'-AATCGAAGGGCAAAGTGGAG-3', reverse 5'-GTTGTGGGATTGGCTGGTAG-3'; PAX2 - forward 5'-TACTACGAAACGGGCAGCAT-3', reverse 5'-ATAGAAGAGACGCTGGGCA-3'.

GAP43 - forward 5'-GTCCTAAAGCAGAAGAGGCC-3', reverse 5'-CGGAGAGTTGTCACTGGAAG-3'; MBP - forward 5'-AGC-CAGGGTGTGGATATGGA-3', reverse 5'-CCTTGATCCCTGTGAGCTGAT-3'; BLBP - forward 5'-CAGGTGGGAATGTGACTAA-3', reverse 5'-TGCACTTCCTATCATCTGGA -3'; HTT - forward 5'-AATCCCGAATTTGTAGGG-3', reverse 5'-AACAGGAAGGGTCATTGCAC-3'; HIP1 - forward 5'-GCAAGGCTGAAAGTTGGAG-3', reverse 5'-CAAAACCCAAAATCCAAATG-3'; MAPK10 - forward 5'-ACTAGGAACACCATGCCAG-3', reverse 5'-AATCCGGAAAGAGCTTGGGG-3'; RAB8A - forward 5'-AGTCTCTGAGGGTGGTTGG-3', reverse 5'-GTACAGTGGATGCATGGGGA-3' and PARK7 - forward 5'-CAAAGGCCTCATAGCTGCTG-3', reverse 5'-TTGCCATCTTTTCCA-CACG-3'.

2.7. Immunohistochemistry

Spinal cords were fixed in 4% paraformaldehyde for 12–24 h at 4 °C followed by 24 h immersion in 30% sucrose for cryoprotection. Lumbar spinal cord segments were cut in 16 µm thin sections using the sliding cryostat Leica CM1850 (Leica Biosystems, Germany). For immunostaining, the tissue sections were first treated with blocking solution (3% normal goat serum, 3% bovine serum albumin, 0.3% Triton X -100 and 10x PBS) for 1 h at RT. After blocking, sections were incubated with primary antibodies overnight at 4 °C. Primary antibodies used were targeting PAX2 (Abcam, ab79389, 1:200) and BLBP (Abcam, ab32423, 1:1000). The corresponding secondary antibodies were conjugated with Alexa Fluor Plus 488 or 555 (1:500 dilution; Invitrogen, USA) and were incubated for 2 h at RT, followed by 20 min incubation with 1 µg/ml solution of 4,6 -diamidino - 2 -phenylindole (DAPI; Thermo Fisher Scientific, USA). Samples were analysed with Olympus IX83 microscope (Olympus, Japan). ImageJ was used for image processing.

2.8. Statistical analysis

For WB, qRT-PCR and immunofluorescence analysis the data are presented as the means ± SD. Statistical analysis was performed using the Student's t-test (GraphPad Prism software). For MS data downstream bioinformatics analysis (two-sample t-test) was performed using the Perseus software package version 1.6.13. The accepted level of significance was $p < 0.05$. $p < 0.001$ Very significant ***, 0.001 to 0.01 Very significant **, 0.01 to 0.05 Significant *, ≥ 0.05 Not significant.

3. Results

3.1. Protein identification and functional classification

Spinal cord tissue samples of P5 and P18 opossums *Monodelphis domestica* were subjected to LC-MS/MS analysis done on an Easy-nLC 1200 coupled to a QExactive HF mass spectrometer. According to the criteria for protein identification, thousands of proteins were identified in two independent biological samples, each in triplicate. We identified a total number of 4735 proteins for two biological samples, among which 1215 proteins (25.66%) were overlapped between both biological samples, with different intensity. The 918 identified proteins were unique for P5 spinal cords, while 714 were unique for P18 spinal cords (the raw data are stored in the UniRi repository; <https://urn.nsk.hr/urn:nbn:hr:193:264020>). The detected proteins were categorized using the UniProt protein knowledge database and PubMed, based on their molecular function, biological process, cellular component and protein class (Supplementary Figs. 1 and 2), revealing differences between P5 and P18 opossum spinal cord protein content. The most informative was the categorization based on the molecular

pathways in which the detected proteins play a part, revealing more than 70 involved pathways for each age group (Supplementary Table 1). Among the 1215 identified proteins differentially distributed in P5 and P18 opossum spinal cords, the abundance of the proteins related to NDD was prominent. These results were in accordance with the previously detected genes differentially expressed between the regenerating and non-regenerating opossum spinal cords [7,8].

In order to reduce the number of the candidate molecules, downstream bioinformatic analysis were performed using the Perseus software package. We noted 615 proteins that had significantly higher expression in P5 spinal cords than in P18 spinal cords, and 600 proteins with significantly higher expression in P18 spinal cords than in P5 spinal cords (Fig. 1A). Pearson correlation coefficients between spinal cords P5 and P18 were 0.881 ($p < 0.05$), implicating good biological reproducibility of the study. Several candidates were chosen for further analysis based on their biological relevance and the overlapping with the previous transcriptomic data [7,8].

3.2. Validation of the selected proteins by Western blot

To validate and confirm the results obtained by MS, the distribution of selected proteins in the opossum P5, P18 and P30 spinal cord tissue was analysed by WB technique. Six proteins, known to be specific cell markers or important in CNS development, were selected to undergo confirmatory tests: Glial fibrillary acidic protein (GFAP; astrocytic marker), Paired box protein Pax-6 (PAX6; CNS development), Paired box protein Pax-2 (PAX2; embryonic development), Growth associated protein 43 (GAP43; neurite outgrowth), Myelin basic protein (MBP; oligodendrocyte differentiation and myelination in developing CNS) and Brain lipid-binding protein (BLBP; marker for radial glia). Western blot data confirmed a distribution pattern detected by MS, demonstrating the higher expression of GFAP, PAX6, GAP43, MBP and BLBP in P18 and P30 spinal cords when compared to P5 and opposite for the PAX2 (Fig. 1B and 1C). P30 spinal cords were used as an additional control for the analysis of protein and gene expression, since at that age neuroregeneration in opossum spinal cord tissue is completely abolished [6]. Our results have shown the match between the MS and WB results for the selected proteins known to be important for the CNS development, indicating the comprehensive relevance of the obtained MS results (Table 1A).

3.3. Validation of the differentially expressed genes by qRT-PCR

To verify if the changes in protein distribution followed the changes in the expression of the related genes, the qRT-PCR transcription analysis was performed for the selected candidates. The results demonstrated the higher expression of 5 genes, including GFAP, PAX6, GAP43, MBP and BLBP in P18 and P30 spinal cords, and the opposite for PAX2 (Fig. 1D), revealing the congruence with the proteomic data.

3.4. Immunohistochemical localization of the differentially expressed proteins

The cellular distribution of two selected proteins, PAX2 and BLBP, was analysed in the opossum spinal cord tissue with and without regenerative capacity, using immunofluorescence (Fig. 2A). The choice of the PAX2 and BLBP proteins was related to the existence of commercially available antibodies complementary to opossum tissue. Furthermore, the PAX2 specific antibody was previously used on opossum primary cortical neuronal cultures [14]. The results have shown the abundance of the PAX2 protein in

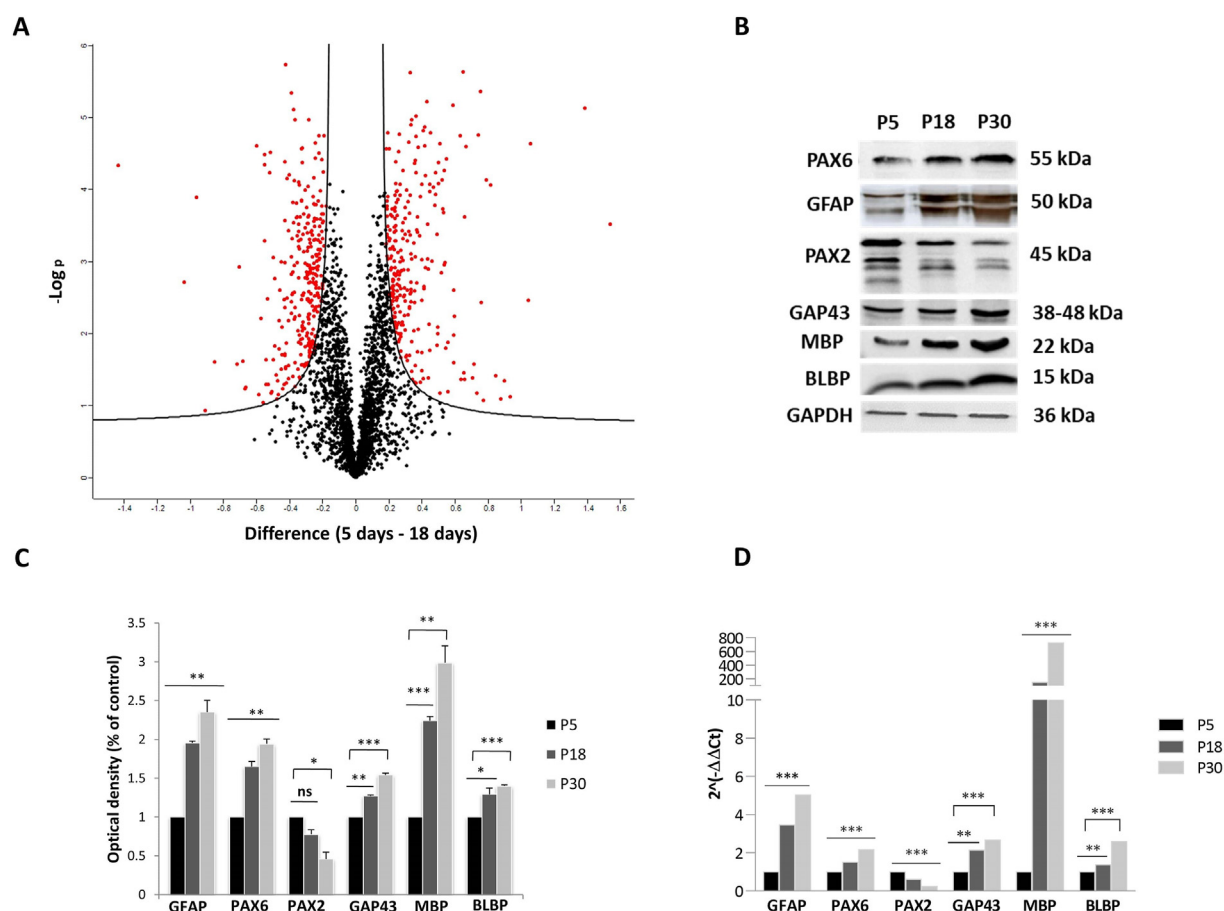


Fig. 1. The MS analysis of the proteins differently distributed in the P5 and P18 opossum spinal cords and the validation of the results by WB and qRT-PCR. (A) Plot of the $-\log p$ -value vs. the $\log_2$ intensity difference of the P5 and P18 opossum spinal cords. The red dots represent the significantly regulated proteins, showing on the left side of the graph the proteins elevated in P5, and on the right side the proteins elevated in P18. The proteins without a significant change in distribution between the samples are shown as the black dots on the graph. (B, C) WB analysis of the selected proteins. On (B) the representative image of the detected WB signal is shown, while the quantification of the relative levels of protein expression is shown on the histogram (C). The qRT-PCR data (D) show the relative level of gene expression for the selected candidates in P5, P18 and P30 opossum spinal cords. The obtained values were normalized to GAPDH and expressed as fold difference compared to control (P5). Data are presented as means $\pm$ SD of three independent experiments. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1

Selected proteins detected by mass spectrometry as differentially distributed in P5 and P18 opossum spinal cords: (A) specific cellular markers or proteins with the established role in CNS development and (B) proteins related to neurodegenerative diseases.

A			
Proteins unique for P5 spinal cords	ID ¹	Gene symbol ¹	LFQ intensity
Paired box 2	F6X9M8	PAX2	7×10^5
Proteins unique for P18 spinal cords	ID	Gene symbol	LFQ intensity
Myelin basic protein	F7GF44	MBP	1.32×10^9
Proteins up-regulated in P18 spinal cords	ID	Gene symbol	Fold change
Paired box 6	F7DII5	PAX6	1.21
Glial fibrillary acidic protein	F6WYV7	GFAP	5.53
Growth associated protein 43	F7BTK7	GAP43	1.68
Fatty acid binding protein	F7ELF5	FABP7 (BLBP)	1.01
B			
Proteins up-regulated in P5 spinal cords	ID	Gene symbol	Fold change
RAB8A, member RAS oncogene family	F7GGI0	RAB8A	1.07
Proteins up-regulated in P18 spinal cords	ID	Gene symbol	Fold change
Huntingtin	F7C6C4	HTT	1.45
Huntingtin interacting protein 1	F6YXY3	HIP1	1.14
Mitogen-activated protein kinase	F6ZA47	MAPK10	1.01
Parkinsonism associated deglycase	F7A9B9	PARK7	1.23

¹ ID and gene symbol according to UniProt protein knowledge database; <http://www.uniprot.org>.

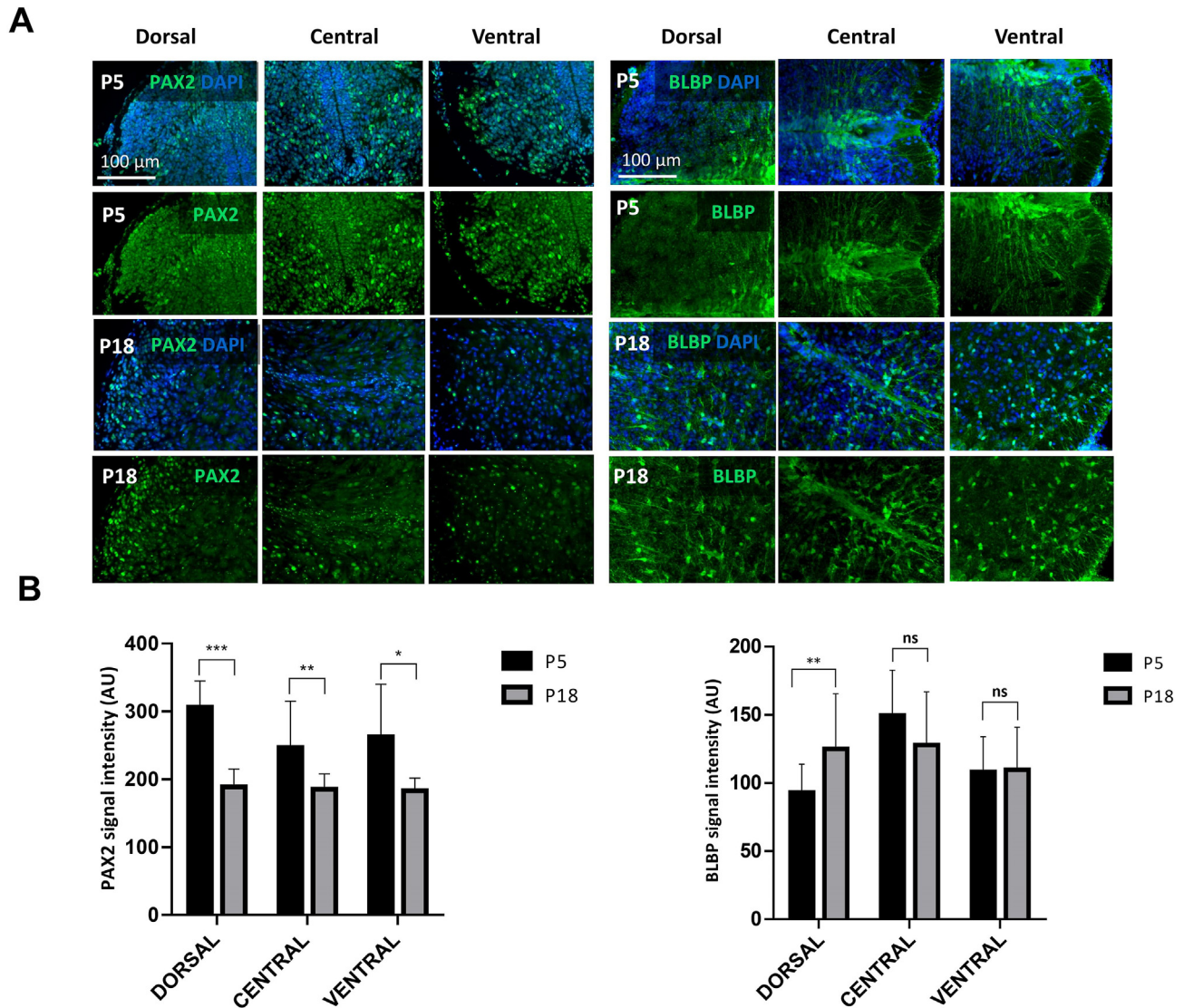


Fig. 2. Immuno-staining for PAX2 and BLBP, differentially expressed in P5 and P18 opossum spinal cords. (A) Spinal cord sections were immuno-stained for PAX2 and BLBP (green signal), while the nuclei of all cells were stained with 4',6-diamidino-2-phenylindole (DAPI; blue signal). In the left part of the figure the representative images of the PAX2 immunostaining are shown, while for the BLBP are shown on the right part. Furthermore, the results for the different parts of the spinal cord (dorsal, central and ventral) are presented. Scale bar, 100 μ m. (B) Bar graphs represent the average immunostaining intensity (Arbitrary Units, AU) in dorsal, central and ventral ROIs (50 $\times$ 50 pixels) of P5 and P18 spinal cords. Data are mean $\pm$ SD from 3 to 5 sections from three different spinal cords for each group. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the gray matter of the opossum P5 spinal cord tissue, with a significant decrease in the P18 tissue, where the remaining signal was mostly present in the dorsal horn. The BLBP positive cells were distributed throughout the spinal cord tissue of both P5 and P18 opossum spinal cords, but with the significant increase in the signal in the dorsal and ventral parts of the P18 spinal cords, as previously observed in rodent spinal cord [15,16]. The BLBP immunosignal that is extending from the central canal through the adjacent gray matter, reaching the outer edge of the white matter, is resembling the representation of the nestin positive radial glial cells [17]. To quantify the immunofluorescent staining, the average intensity of PAX2 and BLBP immunofluorescent signal (expressed in arbitrary units, AU) was measured in selected regions of interest (ROI; 50 $\times$ 50 pixels; Fig. 2B). The results obtained were in accordance with proteomic data, further supporting the biological relevance of the detected proteins.

3.5. Proteins related to neurodegenerative diseases

Based on the molecular pathway classification, the differential representation of the proteins related to neurodegenerative diseases in the opossum spinal tissue with different regenerative abilities was very prominent. Thus, 3.9% of the proteins found to be upregulated in P5 opossum spinal cords were involved in Alzheimer disease (amyloid secretase and presenilin pathway), 2.6% in Huntington disease and 1.3% in Parkinson disease. On the other hand, 1.9% of the proteins upregulated in the P18 opossum spinal cords were involved in Alzheimer disease (amyloid secretase and presenilin pathway), 2.8% in Huntington disease and 0.5% in Parkinson disease. Several candidates were chosen for further analysis: Huntingtin (HTT), Huntingtin interacting protein 1 (HIP1), Mitogen-activated protein kinase 10 (MAPK10; related to Alzheimer's disease [18]), Rab-8A, member of the RAS oncogene family (RAB8A; related to Parkinson's disease [19]) and Parkinsonism

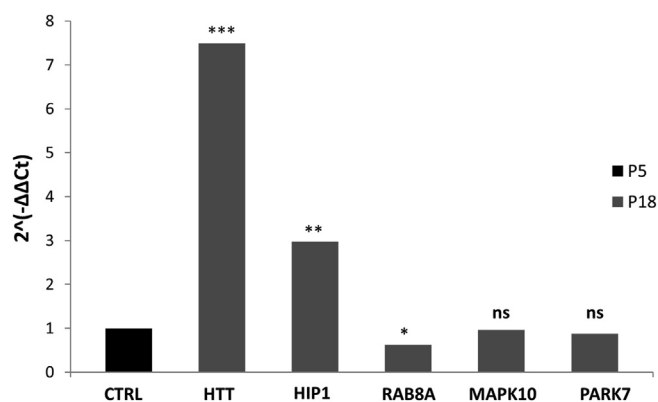


Fig. 3. qRT-PCR analysis of the selected proteins related to neurodegenerative diseases in P5 and P18 opossum spinal cord tissue. The mRNA levels of the Huntingtin (HTT), Huntingtin interacting protein 1 (HIP1), Mitogen-activated protein kinase 10 (MAPK10), Rab-8A, member of the RAS oncogene family (RAB8A) and Parkinsonism associated deglycase (PARK7) were quantified in spinal cord tissue of P5 and P18 opossums. Data are presented as means $\pm$ SD of three independent experiments. Obtained values were normalized to GAPDH and expressed as fold difference compared to control (P5).

associated deglycase (PARK7). The qRT-PCR transcription analysis was performed to investigate the correlation of gene changes with the protein distribution detected by MS (Table 1B). The same pattern of expression as the one detected with MS was observed for the RAB8A (higher expression in P5 spinal cords than in P18), HTT and HIP1 (both with higher expression in P18 spinal cords than in P5), while for MAPK10 and PARK7 no significant difference was detected (Fig. 3). The results have shown the partial overlapping between the pattern of gene expression and protein distribution of the selected candidate molecules related to NDD.

4. Discussion

In this study, we performed the LC-MS/MS analysis to determine the protein profile of the opossum *Monodelphis domestica* intact spinal cords with different regenerative capacities. A total number of 4735 proteins involved in a spectrum of biological functions were identified, revealing the abundance of proteins related to neurodegenerative diseases and the overall concurrence with the previously obtained transcriptomics data.

Comparative proteomic analysis that pinpoint the differences between regenerating and non-regenerating systems were performed in very few research studies, e.g. between rat peripheral sensory and motor nerves [20], after rat spinal cord transection [21] and following transection of opossum neonatal spinal cord [8–10,12]. The main goal of the previous studies was to reveal the cellular response to traumatic SCI, and thus the most prominent protein changes detected in both regenerating and non-regenerating tissues were found to be related to inflammatory response to injury, probably overshadowing the more subtle changes related to neuroregeneration. In this study, the naïve mammalian spinal cord tissues with different regenerative capacities were compared, to allow the detection of a wide spectrum of protein changes during the developmental period when regeneration stops being possible.

Efforts were made to validate the results obtained by comparative proteomics using WB, and qRT-PCR to compare gene expression changes for the selected candidates, revealing the overall congruence of the results, both at the gene and protein level. Moreover, in the previous studies [7,8], the comprehensive changes in mRNA content of developing opossum spinal cord at ages when

regeneration can and cannot occur were identified. The results coincided for the majority of the protein functional groups, but the most interesting was the finding of the myriad of the proteins known to be involved in the pathology of the neurodegenerative diseases. Expression of several interesting genes related to neurodegenerative diseases was analysed to verify the protein changes detected by MS. For the majority of the genes, there was the perfect correlation between the detected protein and gene changes, while for the two of the selected proteins the gene changes were not significant. Although this observation maybe due to the technical limits of the techniques used [22], there is the possibility of the biological discrepancy between the gene and protein levels. In fact, unlike the genome, which is characterized by greater stability, the proteome actively changes in response to various factors, including the organisms developmental stage, as well as internal and external conditions [23]. It is still uncertain how closely levels of mRNAs relate to levels of their corresponding proteins. Two studies [24,25] have explored this problem in mammalian cells, and suggest only a modest correlation.

The results of this study suggest that proteins known to have an important role in the pathophysiology of neurodegeneration in aged CNS, could have an important function during CNS development. It is expected that the database generated in this study will provide a solid basis for further comprehensive investigation of the functional relevance of the selected proteins in the spinal cord development and regeneration.

Declaration of competing interest

Authors declare no conflicts of interests.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bbrc.2021.11.078>.

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