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Expression and cellular localization of the classical progesterone receptor in healthy and amyotrophic lateral sclerosis affected spinal cord

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1. Title Page

Title: Expression and cellular localization of the classical progesterone receptor in healthy and amyotrophic lateral sclerosis affected spinal cord

Running title: PR expression in spinal cord

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2. Abstract

Background and purpose: Previous studies have suggested that elevated progesterone levels are associated with a slower disease course in ALS. Given that the effects of progesterone are mediated in part by the classical progesterone receptor (PR), we have examined the expression and cellular localization of the A and B isoforms (PR-A and PR-B, respectively) of the PR in control (neuropathologically normal) and ALS-affected spinal cord (SC).

Methods: We performed semi-quantitative RT-PCR, immunohistochemistry (IHC) and immunofluorescence (IF) in the cervical and lumbar SC of post-mortem ALS patients (n=19) and control subjects (n=10). Primers and antibodies used allowed the detection of both PR-A and PR-B isoforms together (PR-A+B) or PR-B isoform alone.

Results: Lumbar PR-A+B and cervical PR-B mRNA expression were significantly higher in ALS than controls. PR-A was inferred to be expressed at higher levels in the lumbar and at lower levels in the cervical SC of ALS patients compared with controls. In both ALS and controls, PR-A+B immunoreactivity (IR) was occasionally detected in motor neurons. In contrast, PR-A+B IR was prominent in axonal processes and vessels. This was more evident in nerve roots and large arteries in ALS compared with controls. We also observed colocalization of PR-A+B with markers of neurons, axonal processes and vascular endothelium.

Conclusions: We provide evidence that both PR-A and PR-B isoforms are expressed in the human SC, with some regional variation in isoform expression between ALS and controls. The IR was more prominent in nerve roots and large arteries in ALS, suggesting a potential role in the degenerative process.

3. Keywords: progesterone receptor, neuroprotection, progesterone, spinal cord, ALS.

4. Main text

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset neurodegenerative disease in which motor neurons are selectively targeted. Several contributing factors have been identified in the past two decades [1-4], but its cause still remains elusive. There is evidence that gender and hormonal status influence clinical features of ALS [5] with incidence and prevalence being greater in men than in pre-menopausal women [6-8]. In line with this, progesterone (PROG) is associated with neuroprotective, pro-myelinating and anti-inflammatory effects in the nervous system [9-11]. PROG can be synthesized de novo in the nervous system and consequently is considered a neurosteroid [9]. Furthermore, two clinical trials have recently demonstrated the benefit of PROG in traumatic brain injury (TBI) patients, mainly in terms of a lower mortality rate and a better outcome than those randomized to placebo [12,13].

PROG neuroprotection has also been shown in the Wobbler model of motor neuron degeneration where it decreases oxidative stress and mitochondrial membrane disruption in motor neurons, increases neuronal brain-derived neurotrophic factor (BDNF) mRNA, restores choline acetyl transferase (ChAT) enzymatic activity and immunoreactivity in the spinal cord, prevents motor neuron vacuolation and increases muscle strength and mouse survival [14-16]. PROG effects are mediated by multiple progesterone receptors (PR) that include the nuclear PR-A and PR-B receptors, the membrane receptors mPRs and the progesterone receptor membrane component 1 (PGRMC1) [17,18].

While several studies have addressed the presence and isoform characterization of the nuclear PRs in human non-reproductive tissue [19-21], whether they are expressed in the human spinal cord is unknown. We have observed both neuronal and non-neuronal reactivity of PR-A and PR-B in ALS and control spinal cord, and that PR-B expression in particular is elevated in ALS. These observations suggest a potential role of PR expression in the disease process of ALS, a role which we postulate may reflect a neuroprotective action.

Material and Methods

Cases

All ALS cases were both clinically and neuropathologically confirmed using the El Escorial Criteria (World Federation of Neurology Research Group on Neuromuscular Disease, 1994). Written consent for tissue donation at autopsy was obtained from either the patient (ante-mortem) or spouse (post-mortem) in accordance with the London Health Sciences Centre ethics policies. Cervical and lumbar spinal cords were obtained from control subjects with no history of motor neuron disease or evidence of neuropathology (n=10) and from patients with sporadic ALS (n=19). Genotypic analyses were performed in all patients for C9orf72, SOD1, TARDBP and FUS/TLS (R. Rademakers, Mayo Clinic, Florida). One case was determined to carry *mt*TDP-43 (3'UTR 81-84 del CATA) and 4 cases demonstrated a repeat expansion in C9orf72 (Table 1). Age at death of control subjects ranged from 55-82 years (mean ± SEM, 69.5 ± 3.1 years), males (n=5) and females (n=5); and of ALS patients ranged from 49-85 years, (65.8 ± 2.4 years), males (n=10) and females (n=9). The menopausal status of women with ALS was unknown.

RNA isolation and semi-quantitative RT-PCR

Frozen (-80°C) tissue was available for a subset of both control (cervical n=3, lumbar n=3) and ALS cases (cervical n=6, lumbar n=6). Total RNA was isolated using Trizol reagent (Life Technologies, Burlington, ON, Canada) according to the manufacturer's protocol. cDNA synthesis was performed using 2 µg of total RNA and the Superscript II Reverse Transcriptase system following the manufacturer's protocol (Life Technologies, Burlington, ON, Canada). Semi-quantitative RT-PCR was performed for 35 cycles (94°C for 30s; 58°C for 45s; 72°C for 60s) using primers designed to detect either both human PR isoforms, PR-A+B (Genbank: NM_000926; For: 5'-GAA GAA ATG ACT GCA TCG-3'; Rev: 5'-TCC AGT GCT CTC ACA ACT C-3'), or for PR-B alone (GenBank: NM_000926; For: 5'-CTG TGT CGC CCA GCC GCA-3'; Rev: 5'-CGG GGC CAA ACA GGC ACC-3'). PR-B isoform primers were designed for a fragment in the coding region unique to PR-B; whereas, since PR-A's coding region is included in PR-B's, the PR-A+B set of primers could only pick up a common fragment. As an internal control, all samples were analyzed for 18S expression (For: 5'-AGT TGG TGG AGC GAT TTG TC-3'; Rev: 5'-TTC CTC GTT CAT GGG GAA TA-3') using 15 cycles (94°C for 45s; 58°C for 45s; 72°C for 60s). Samples were separated by agarose gel electrophoresis and visualized using ethidium bromide. The gels were subsequently quantified using Image J software. All experiments were performed in triplicate. Positive controls for PCR reactions consisted of PR-A+B and PR-B cDNA fragments cloned into pGEMT-Easy plasmid (Clontech Laboratories Inc., Mountain View, CA, USA). The Student's *t*-test was used to determine significant differences between the two groups.

Immunohistochemistry and light microscopy

Six µm thick sections were prepared from formalin fixed and paraffin embedded cervical and lumbar spinal cords from control and ALS patients. Sections were deparaffinized using standard protocols. Antigen retrieval was performed using sodium citrate buffer (10mM sodium citrate, 0.05% Tween 20, pH 6; 2100 Retriever chamber) and sections were immunolabeled overnight at 4°C using rabbit polyclonal anti-PR-A+B (1:100; Dako Canada Inc., Burlington, ON, Canada) or anti-PR-B (1:50; Neomarkers, Fremont, CA, USA). After a wash with PBS, sections were incubated with a biotinylated secondary antibody (1/200 dilution, 60 min). The antigen-antibody complex was visualized using the Vectastain Elite ABC kit (Vector, Burlington, ON, Canada). Colorimetric detection was performed using nickel 3,3'-diaminobenzidine (0.15 mg/ml in 0.03% H_2O_2; Sigma-Aldrich, Oakville, ON, Canada), followed by counterstaining with Nuclear Fast Red and analyzed with an Olympus BX45 light microscope and Image-Pro Plus software (Leeds, Minneapolis, MN, USA). Positive control tissues consisted of mouse uterus including endometrium, myometrium and placenta at gestational day 12. Omission of the primary antibody was used as a negative control. The specificity of the anti-PR-A+B (Dako) and the anti-PR B (Neomarkers) has been previously validated [22]

Immunoreactivity scoring

Images from a subset of control (n=7) and ALS cases (n=8) taken from motor neurons and axonal processes in ventral horns, axonal processes in ventral roots and large extra spinal arteries, were analyzed semi-quantitatively for the expression of PR-A+B. Scoring of immunoreactivity (IR) levels was done according to a previously reported method of intensity scoring [23]. An IR intensity score between 0 and 4 was

assigned based on IR levels in these structures. A score of 0 indicated the absence of staining whereas a score of 4 indicated an intense IR. Scores were assigned by a blinded investigator (G.M.G-M) for all images of PR-A+B IR (Supplementary Figure 1).

Immunofluorescence and confocal microscopy

Six µm paraffin embedded spinal cord sections were dried overnight and then treated for 15h with 365nm UV light to decrease lipofuscin-induced autofluorescent signal [24]. Antigen retrieval was performed by exposing to 10 mM sodium citrate, 0.05% Tween-20, pH 6 in a 2100 Retriever chamber, followed by incubation with various mouse monoclonal primary antibodies. Phosphorylated high molecular weight neurofilaments were labeled with SMI-31 (1:25000; Sternberger, Lutherville, MD, USA), neurons with anti-neuronal specific enolase (NSE 1:50; Chemicon, Temecula, CA, USA), astrocytes with anti-glial fibrillary acidic protein (GFAP, 1:500; Pharmingen, San Diego, CA, USA) and endothelium with anti-Von Willebrand Factor (VWF 1:50, Abcam, Cambridge, MA, USA). After washing in 1X PBS, the appropriate fluorescent conjugated secondary antibody (1:1000; Alexa Fluor, Life Technologies, Burlington, Canada) was applied for 2h at RT. Nuclear counterstaining was performed using Hoechst 33342. Sections were mounted using Immu-Mount (Thermo Scientific, Kalamazoo, MI), and photographed using an LSM 510 Meta Confocal Imaging System (Carl Zeiss Canada Ltd., Toronto, ON).

Results

PR-A and PR-B mRNAs are up-regulated in ALS spinal cord

Two PR isoforms have been previously described, PR-A and PR-B [25], derived from transcripts at two different start codons and controlled through two distinct promoter systems within the PR gene [26]. The PR-B isoform is a full-length receptor, whereas the PR-A isoform lacks a fragment in the N terminus resulting in a shorter protein, the sequence of which is identical to that portion of the PR-B sequence. This presents a unique issue for this study, as only PR-B can be recognized separately by primers and antibodies while PR-A cannot be separated from the sequence/epitopes of PR-B.

We performed semi-quantitative RT-PCR of PR-A+B and PR-B to examine the PR mRNA expression at the cervical and lumbar levels of the spinal cord in control subjects and ALS patients. In the cervical segment, we observed no significant difference in the expression levels of PR-A+B mRNA between ALS and controls (Figure 1A). However, the expression of PR-B mRNA at the cervical level was significantly higher in patients compared to controls (Figure 1B; $p=0.02$). In the lumbar segment, we observed that the expression of PR-A+B mRNA was significantly elevated in ALS spinal cords (Figure 1C; $p=0.04$). While the expression of PR-B mRNA from ALS lumbar cord was 2-fold that of controls, this did not reach significance (Figure 1D).

These results demonstrate that both PR-A and PR-B isoforms are expressed in the human spinal cord. In addition, our results suggest that the expression of the PR-B mRNA is increased in ALS. While it can be inferred that PR-A mRNA expression levels are reduced in ALS cervical cord and increased in the lumbar cord compared to controls, given the respective levels of PR-A+B, we cannot ascertain this with certainty given the nature of the RNA sequences.

PR-A and -B are present in different structures of the spinal cord

Having observed both PR-A and -B expression in the human spinal cord, we next determined which cell populations were expressing PR. PR-A+B was most prominent in both large and small caliber vessels with staining localized to both endothelial and vascular smooth muscle cells (VSMC) (Figure 2A, D, G and J). While the majority of IR was nuclear, faint cytoplasmic staining was also noted.

In the cervical and lumbar regions of the ventral spinal cord of both ALS patients and controls, some but not all motor neurons were immunoreactive for PR-A+B. In those motor neurons in which IR was evident, we observed weak cytoplasmic and stronger axonal IR. The predominant staining pattern in both the cytoplasm and axonal processes was a diffuse granular pattern in ALS spinal cords (Figure 2B cervical, 2H lumbar) and controls (Figure 2E cervical, 2K lumbar).

The mouse uterus was used as a positive control, and its distinct nuclear localization was readily detected in the endometrium, endometrial glands, stroma, and placental tissues (Supplementary Figure 1). This nuclear staining pattern was not evident in the human spinal cord samples in motor neurons. Positive nuclear IR was detected in cells that are thought to correspond to glial cells (Figure 2B, *black arrows*).

We observed intense IR for PR-A+B in the ventral nerve roots, where we detected prominent staining in axons as well as encircling myelin sheaths in ALS patients (Figures 2C cervical, 2I lumbar). Control subjects had a weaker IR limited to axons (Figures 2F cervical, 2L lumbar). Negative controls (lacking primary antibody) of all these structures can be seen in Figures 2M through 2O.

PR-B IR was also detected in motor neurons and axonal processes of human spinal cord (Figure 3). The staining was predominantly nuclear both in glial cells (Figure 3A, *black arrows*) and VSMC (Figure 3D, *black arrows*); while motor neurons showed a granular cytoplasmic staining both in ALS (Figure 3A) and control samples (Figure 3B). In contrast, there was no IR in ventral nerve roots (Figure 3C).

We also performed a semi-quantification of PR-A+B IR (see Supplementary Figure 2 for the scoring scale). The staining intensity of motor neurons and axonal processes was similar between ALS patients and controls; however, ventral roots showed increased IR in ALS cases compared to controls. Similarly, vessels revealed higher scoring among ALS patients, regardless of gender (Table 2). It is of note that we detected no difference in IHC between sporadic patients or those with hexanucleotide expansion of C9orf72 or with TDP43 mutations.

These results show that PR-A and PR-B are present in the human spinal cord, particularly in the vasculature, ventral root axons and nuclei of glial cells. Weak IR was found in motor neurons, predominantly in the cytosol and not significantly different from controls. Furthermore, PR IR in nerve roots and large arteries tends to be more intense in ALS spinal cords.

PR-A and -B colocalize with axonal processes and vessel endothelium in spinal cord

We next performed confocal microscopy in order to more clearly define the cell populations expressing PR. Using anti-NSE, a recognized neuronal marker, we observed that the few motor neurons with PR-A+B IR were also NSE positive (Figure 4C). Using anti-SMI-31 (recognizing phosphorylated high molecular weight neurofilaments), we

observed strong colocalization with PR-A+B in ventral root axons (Figure 4F) and to a lesser degree those within the white matter axons. At the periphery of the axons, PR-A+B positive and SMI-31 negative immunofluorescence was also observed, suggesting PR expression in the myelin sheaths or in the cytoplasm of Schwann cells. (Figure 4F, *white arrow*). Using a marker of endothelial cells, Von Willebrand Factor, we confirmed PR-A+B expression in the endothelium of both large arteries and small arterioles (Figure 4I, *white arrowhead*). In a high magnification image of an arteriole, a pericyte-like cell adjacent to the endothelial lining showed PR-A+B reactivity (Figure 4I, *white arrow*). In terms of the astrocytic marker GFAP (glial fibrillary acid protein), none of the images showed clear colocalization with PR-A+B (data not shown).

These results clearly show the colocalization of PR-A+B in distinct cell types in axonal processes and vessel endothelium of the spinal cord.

Discussion

We have described for the first time the presence of the two isoforms of the PR in the human spinal cord. Furthermore, some variations in PR isoform expression were obtained when post-mortem spinal cord samples from control and ALS patients were compared.

There is a clear trend for increased expression of PR-B in cervical and lumbar ALS spinal cord, although only the cervical values meet statistical significance. PR-A+B mRNA levels were higher in the lumbar spinal cord of ALS patients, and when taken with the data of lumbar PR-B expression, also suggests that PR-A is expressed at higher levels in the lumbar cord.

We found PR-A+B cytosolic IR in motor neurons of the human spinal cord ventral horn, and more predominant IR in the axonal processes of the ventral nerve roots. In contrast, immunocytochemistry in the rat spinal cord has demonstrated that neurons localized in the ventral horn, in addition to glial cells of the gray and white matter and ependymal cells, were PR positive not only in the cytoplasm but also in the nucleus [27,28]. Evidence of cytoplasmic PR in the human spinal cord may suggest extra nuclear mechanisms of hormone action. The presence of extra nuclear PR has also been reported in the pre- and post-synaptic structures in the rat hippocampus, which may be linked to the control of neuronal excitability and synaptic plasticity [29].

The colocalization of PR-A+B with the neuronal markers, especially SMI-31, strongly suggests that the PR may have an as yet unknown role in these cells. It has been reported that PROG along with its reduced metabolites dihydroprogesterone (DHP) and tetrahydroprogesterone (THP), are neuroprotective factors in peripheral nervous system injury models. These include experimental diabetic neuropathy [30], hereditary neuropathy [31] and nerve transection or sciatic crush injury [32,33]. Moreover, PROG and DHP may promote nerve repair after injury by reducing axonal supernumerary sprouts and increasing their diameter, by affecting enzymatic activity of the Na^+, K^+ ATPase pump and by influencing the expression of the peripheral myelin proteins P0 and PMP22 [33]. The finding that PROG and DHP are able to interact with the PR might suggest a role for this classical steroid receptor on nerve repair [34].

In addition, the IR of PR-A+B in nerve roots of the human spinal cord involved staining of the axons and myelin. The immunostaining of these structures was more intense in ALS patients compared to controls, which could suggest a similar role in nerve repair that was demonstrated in crush injury [33]. Preservation of axonal integrity in ALS

may be of utmost importance, considering the hypothesis of retrograde ‘‘dying-back’’ degeneration [35,36] involving abnormalities in retrograde axonal transport leading to motor neuron death, which supports a peripheral origin for factors initiating the onset of this disease [37-39]. Indeed, Fischer and colleagues [36] have demonstrated initial denervation at the neuromuscular junction, followed by severe loss of motor axons from the ventral root and finally loss of motor neuron cell bodies from the spinal cord that coincides with clinical manifestations of the disease in a mutant SOD1 (G93A) ALS mouse model.

The presence of the PR in myelin sheaths would be in agreement with previous reports that have described the synthesis of PROG and the PR in Schwann cells of the sciatic nerve [34]. In addition, treatment with a PR and glucocorticoid receptor antagonist (RU 38486) produced a significant reduction of axon diameter in parallel to an increase in neurofilament density, suggesting that PROG and/or glucocorticoid signals are not only involved in the control of the myelin compartment but also in axonal maintenance [40].

We also observed PR-A+B localized in vessels in the spinal cord, both in endothelial cells and in VSMC. Interestingly, colocalization of PR-A+B with a marker of endothelium (Von Willebrand factor) was also evident in small and large arteries, probably indicating a role for this receptor in vascular physiology in the human CNS. The PR has been shown to be expressed in blood vessels by several types of endothelial cells [41] and by VSMC of the tunica media [42-44]. There are conflicting reports as to the activity of the PR in VSMC, with it being suggested that PROG enhances proliferation, migration, and apoptosis of VSMC *in vitro* [45] and *in vivo* [46]; while others report an *in vitro* role in inhibition of cell proliferation and migration [46,47]. Indeed, extra-nuclear

activation of the PR through non-genomic mechanisms has recently been demonstrated on the basis of PROG's anti-proliferative effects on VSMC [41,47]. It is agreed, however, that PROG diminishes endothelial cell proliferation via genomic PR actions, and stimulates vasodilation via nitric oxide production [41,45].

The role of increased expression of the PR in large arteries of the ALS-affected spinal cords will have to be elucidated through further study. Nevertheless, endothelial and VSMC localization of PR was evident for both experimental groups. The overall role of blood vessels in the pathogenesis of ALS is poorly understood. Oosthuyse et al [48] have shown that the deletion of the hypoxia-response element in the vascular endothelial growth factor (VEGF) promotor (that generated VEGFδ/δ mice) caused ALS-like progressive motor neuron degeneration. Other authors report a disruption in the blood-spinal cord barrier in SOD1 G93A transgenic mice as a consequence of a reduction in the levels of tight junction proteins between endothelial cells [49]. These authors showed, in the presence of mutant SOD1, that endothelial damage accumulated prior to motor neuron degeneration supporting a possible central contribution to disease initiation.

While the role of the PR in ALS remains to be clarified, it is clear that PR-A and PR-B are expressed in the human spinal cord, and that expression in ALS-affected spinal cord is not identical to that seen in control. This suggests a role for the PR and PROG in some facet of the disease -likely in a repair attempt given the neuroprotective role of PROG reported elsewhere. The function of the classic nuclear PR in vascular structures, neurons and glia in the spinal cord should be the subject of future studies to clarify if PROG is neuroprotective in ALS as may be inferred by previous studies in other neurodegenerative and injury models.

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7. Table and figure legends

Table 1. Demographic characteristics of patients and control subjects.

Table 2. Semi-quantification of PR IR according to region and gender. Semi-quantification of PR-A+B IR assessing a randomly selected subset of patients (controls n=7 and ALS n=8). See Supplementary Figure 2 for the scoring scale. Ventral roots and large arteries showed increased IR in ALS cases compared to controls.

Figure 1. Relative expression of PR-A+B and PR-B mRNA in spinal cord (SC). (A, C) Semi-quantitative PR-A+B expression in the cervical and lumbar SC, respectively. PR-A+B mRNA is significantly higher in the lumbar segment of the SC of ALS patients compared with controls (*p=0.04). (B, D) PR-B expression in the cervical and lumbar SC, respectively. PR-B mRNA is significantly higher in the cervical segment of the SC of ALS patients compared with controls (* p=0.02).

Figure 2. Immunohistochemistry of PR-A+B. (A-F) show photomicrographs taken from the cervical spinal cord from ALS (A-C) or controls (D-F). (G-L) show photomicrographs taken from the lumbar spinal cord from ALS (G-I) and controls (J-L). Typical photographs were taken of large arteries, motor neurons and ventral roots. (M-O) are negative controls (omission of the primary antibody). Positive nuclear IR was detected in cells that may correspond to glial cells (Figure 2B, *black arrows*). Intensely immunoreactive large arteries and ventral roots were observed in ALS tissues compared

to controls (G versus J, I versus L). Nuclear counter-staining with Nuclear Fast Red. Scale bar = 20µm.

Figure 3. Immunohistochemistry of PR-B. (A) Anterior horn motor neuron in the cervical ALS affected spinal cord. Note the granular cytoplasmic staining and the nuclear immunoreactivity of surrounding cells probably of glial origin (*black arrows*). (B) Anterior horn motor neurons in the control lumbar spinal cord of a control subject with granular cytoplasmic staining and evidence of immunoreactivity in an axonal process (*black arrow)*. (C) Absence of staining in axons and myelin sheaths in the ALS affected ventral root at the cervical level. (D) Spinal artery from the ALS affected cervical spinal cord with predominant nuclear reactivity in endothelial cells and vascular smooth muscle cells. (A-D) Nuclear counter-staining with Nuclear Fast Red. Scale bar = 20µm.

Figure 4. PR-A+B double immunofluorescence staining the in spinal cord. Confocal microscope images showing representative staining patterns of PR-A+B (green, Alexa-Fluor-488) and NSE (neuronal specific enolase), SMI-31 (phosphorylated high molecular weight neurofilament) and VWF (Von Willebrand Factor) (each in red, Alexa-Fluor-568). (A-C) NSE immunoreactive neuron of the anterior horn colocalized with cytoplasmic PR-A+B. (D-F) SMI-31 positive axons of the ventral root colocalized with PR-A+B, and myelin sheaths surrounding axonal processes that were immunoreactive for PR-A+B (*white arrow* in F). (G-I) VWF marker of endothelial cells colocalized with PR-A+B (*white arrowhead* in I). Note autofluorescence arising from intraluminal blood cells in all the tested confocal microscope channels. A vascular smooth muscle cell with

pericyte morphology (*white arrow* in I) showed PR-A+B reactivity. Nuclear counter-staining with Hoescht. Scale bar = 10 µm.

Supplementary Figure 1. Immunohistochemistry of the positive control mouse uterus at gestational day 12. The first row shows immunoreactivity for the PR-A+B antibody (Dako) and the second row PR-B antibody (Neomarkers). The last row corresponds to the negative control (omission of the primary antibody). Nuclear counter-staining with Nuclear Fast Red. Scale bar = 50 µm.

Supplementary Figure 2. Immunoreactivity scoring. Scoring of immunoreactivity levels in different structures of the spinal cord. As demonstrated here, 0 indicates a complete absence of cellular immunoreactivity, whereas a score of 4 indicates an intense immunoreactivity. Scores of 1–3 indicate increasing intensity of immunoreactivity. Nuclear counter-staining with Nuclear Fast Red. Scale = 50µm.

8. Tables

Table 1. Demographic characteristics of patients and controls

#	Group	Region	Gender	Age at death	Used	Mutation Status
1	Control	Cervical	F	75	RT-PCR	NA
2	Control	Cervical	M	67	Semi-Q and RT-PCR	NA
3	Control	Cervical and Lumbar	F	62	RT-PCR	NA
4	Control	Lumbar	M	68	RT-PCR	NA
5	Control	Lumbar	M	74	RT-PCR	NA
6	Control	Cervical	M	82	Semi-Q	NA
7	Control	Cervical and Lumbar	F	55	Semi-Q	NA
8	Control	Cervical	F	82	Semi-Q	NA
9	Control	Lumbar	F	30	Semi-Q	NA
10	Control	Lumbar	M	61	Semi-Q	NA
11	ALS	Cervical	M	68	RT-PCR	C9orf72
12	ALS	Cervical	F	65	RT-PCR	C9orf72
13	ALS	Cervical	M	68	RT-PCR	-
14	ALS	Cervical	F	64	Semi-Q and RT-PCR	-
15	ALS	Cervical	F	68	RT-PCR	-
16	ALS	Cervical	M	74	RT-PCR	-
17	ALS	Lumbar	F	73	RT-PCR	-
18	ALS	Lumbar	M	80	RT-PCR	-
19	ALS	Lumbar	M	75	RT-PCR	TDP43*
20	ALS	Lumbar	F	49	RT-PCR	-
21	ALS	Lumbar	M	69	RT-PCR	-
22	ALS	Lumbar	F	60	RT-PCR	-
23	ALS	Cervical	M	61	Semi-Q	-
24	ALS	Cervical	M	55	Semi-Q	C9orf72
25	ALS	Cervical	M	74	Semi-Q	-
26	ALS	Lumbar	F	61	Semi-Q	-
27	ALS	Cervical	F	64	Semi-Q	C9orf72
28	ALS	Cervical	F	85	Semi-Q	-
29	ALS	Lumbar	M	39	Semi-Q	-

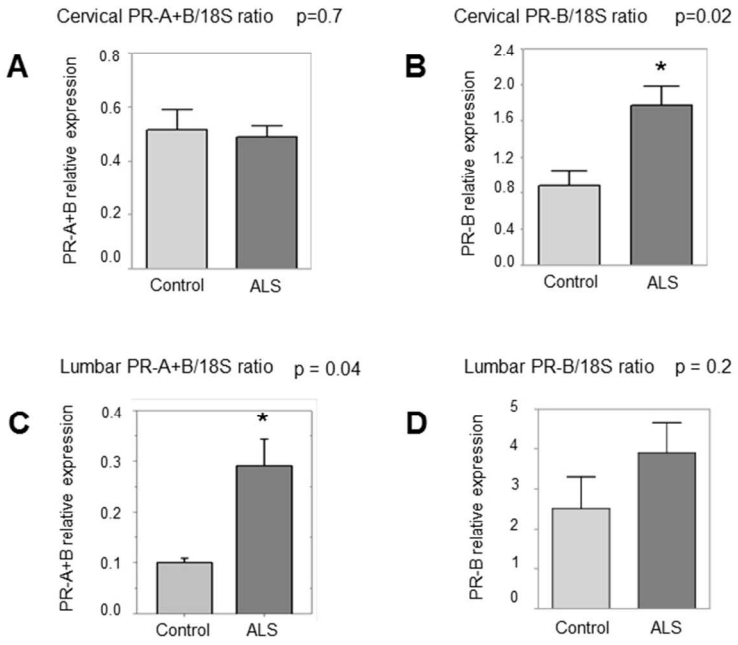
ALS: Amyotrophic Lateral Sclerosis. Semi-Q: semi-quantification

*3'UTR 81-84 delCATA

Table 2. Semi-quantification of PR IR according to region and gender

Group	Region	Gender	Motor neurons	Axons	Nerve roots	Large arteries
Control	Cervical	F	0	0	2	2
		F	1	1	1	3
		M	0	0	1	2
	Lumbar	F	0	2	2	2
		F	2	3	1	2
		M	0	0	3	2
		M	0	0	2	2
ALS	Cervical	F	0	0	2	3
		F	0	2	1	2
		M	0	1	2	3
		M	1	2	4	3
		M	0	0	2	2
	Lumbar	F	0	0	2	4
		F	0	0	4	4
		M	2	2	4	3

ALS: amyotrophic lateral sclerosis



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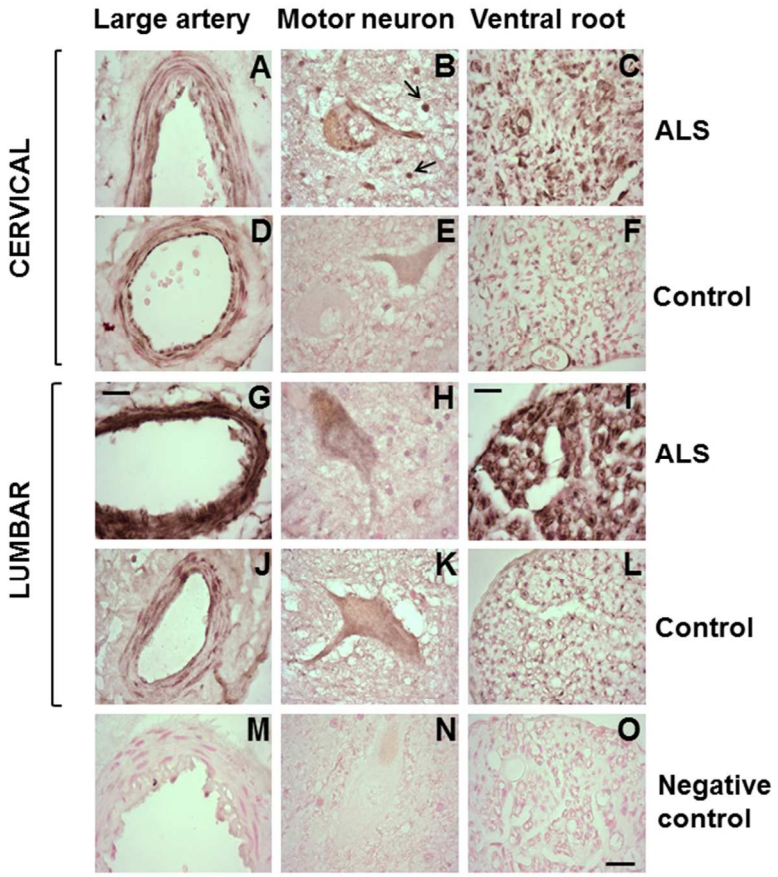

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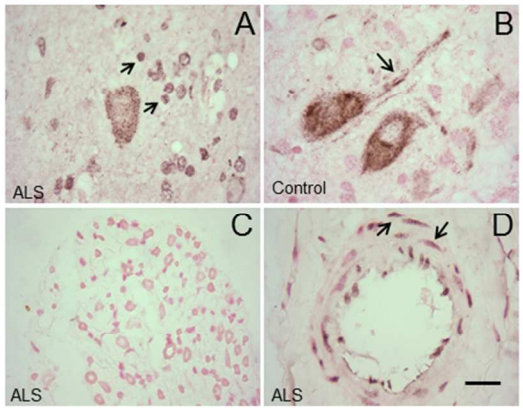

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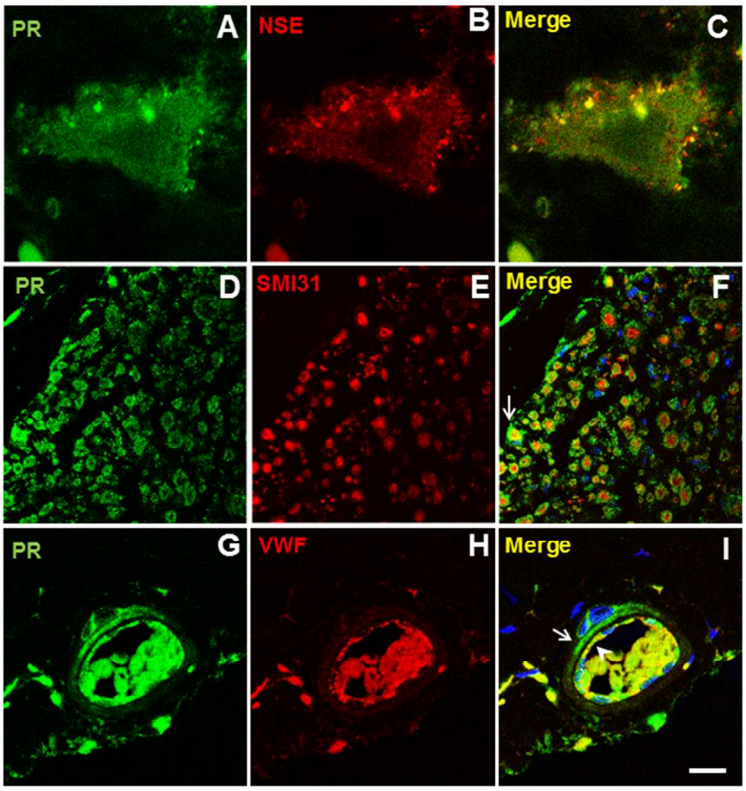

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