PICK1 expression in reactive astrocytes within the spinal cord of ALS rats

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Running title: PICK1 in astrocytes during progression of ALS

Abstract

Aims: The protein interacting with C kinase 1 (PICK1), a PDZ domain containing protein mainly expressed in the central nervous system, interacts with the glutamate receptor subunit GluR2, with the glutamate transporter GLT-1b and with the enzyme serine racemase. These three proteins appear as key actors in the glutamate-mediated excitotoxicity associated with amyotrophic lateral sclerosis (ALS), in both patients and animal models of the disease. In this study, we examined the expression of PICK1 in the spinal cord of transgenic rats expressing a mutated form of the human superoxide dismutase 1 ($hSOD1^{G93A}$) during the progression of the disease.

Methods: Expression of PICK1 was examined by real time qPCR at presymptomatic and symptomatic stages as well as at end-stage. The expression of PICK1 in the different cell types of the spinal cord was examined by immunohistochemistry.

Results: The overall expression of PICK1 is not modified in cervical and lumbar spinal cord of transgenic ($hSOD1^{G93A}$) rats during the progression of the disease. Nonetheless, immunohistochemical studies of lumbar ventral horns revealed a shift of PICK1 expression from motor neurons in healthy rats to activated astrocytes in end-stage $hSOD1^{G93A}$ animals.

Conclusions: Considering the documented influence of PICK1 expression on D-serine release and glutamate transport in astrocytes, these findings point to a potential implication of PICK1 in the progression of ALS.

Introduction

Mainly expressed in the central nervous system (CNS) and testes [1], the protein interacting with C kinase 1 (PICK1) has been extensively characterized for its interaction with AMPA receptor subunit GluR2 and the regulation of calcium-impermeable AMPA receptor trafficking, a fundamental mechanism of synaptic plasticity [2]. However, this unique protein, characterized by the simultaneous presence of a protein-protein interaction domain (PDZ domain, post synaptic density-95 (PSD-95)/Disc large/Zonula occludens-1) and a protein-lipid interaction domain (BAR domain, Bin/Amphyphisin/Rvs), is a scaffold protein controlling the trafficking or the activity of more than 40 proteins in mammals, ranging from neurotransmitter receptors or transporters to enzymes or channels within the CNS (reviewed in [3]. Despite the apparent lack of obvious neurological symptoms in PICK1 knockout mice [4], recent studies, providing evidence for a role for PICK1 in the establishment of drug-seeking behaviours, chronic pain or excitotoxic neuronal death, lead to reconsider the involvement of this protein in neurological disorders [5-9].

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by the progressive and selective loss of motor neurons. Even though the exact mechanisms underlying the pathology remains largely unknown, the glutamate-induced excitotoxicity appears as a common feature of the disease in human as well as in transgenic animal models of inherited ALS [10,11]. Indeed, elevated extracellular glutamate levels have been documented in tissue samples from ALS patients and transgenic rodents [12] and multiple glutamatergic targets, such as the AMPA receptor subunit GluR2, the glutamate transporter-1b variant (GLT-1b) or the NMDA receptor co-agonist D-serine, appear altered during the pathology in human or in animal models (see Table 1) [13-20].

Interestingly, the review of the recent literature on the biochemical properties of AMPA receptor, GLT-1b and serine racemase (SRR, converting L-serine into D-serine) led us to

notice that these 3 proteins share the common feature to physically interact with PICK1 [21-23]. Moreover, several other PICK1 interacting partners, outside the glutamatergic system, were proposed to play a role in the development or progression of ALS (see Table 1). Altogether, these data suggest a role for PICK1 in ALS. In this study, PICK1 expression has been evaluated in the cervical and lumbar spinal cord of a transgenic rat model of ALS (over-expressing the human superoxide dismutase 1 mutated in G93A ($hSOD1^{G93A}$) rats) by quantitative PCR. The disease was characterized by evaluating the expression of specific markers of motor neurons or activated glia and the localization of PICK1 in these cells was examined by immunohistochemistry on spinal cord sections.

Material and methods

Transgenic rats and disease monitoring

The strain of transgenic Sprague-Dawley rats carrying the human SOD1 gene with the G93A mutation initially obtained from Taconic Farms Inc. (New York, NY) were kindly provided by Dr R. Pochet (Université Libre de Bruxelles, Belgium). All experiments performed on adult rats were conducted on female heterozygous animals, all of which were genotyped immediately after birth [24]. Rats were maintained at 21°C with a relative humidity of 55% and 12 h light/ 12 h dark cycle. Food and water were supplied *ad libitum*. All animal procedures were conducted in strict adherence to the European Community Council directive of November 24, 1986 (86-609/EEC), and the Decree of October 20, 1987 (87-848/EEC).

To evaluate the progression of the disease, $hSOD1^{G93A}$ rats were monitored daily between post-natal day 80 and the end-stage by measuring body weight and motor score. Motor score is a global behavioral scoring evaluating motor function in rats modeling ALS initially described by Matsumoto et al. [25]. Disease onset was predicted by the appearance of one paralyzed hind-limb. Only female rats showing initial paralysis at hind-limb were included in this study.

RNA extraction and gene expression analysis

For RNA extraction from spinal cord sections, rats were killed by carbon dioxide asphyxiation and further decapitation. Rats were sacrificed at different stage : presymptomatic (120 days), onset (approximately 155 days) and end-stage (approximately 195 days) [26].Cervical and lumbar spinal cords were carefully isolated from animals and homogenized in Tripure (Roche Applied Science, Vilvoorde, Belgium) using a Teflon potter. Total RNA extraction was performed following the manufacturer protocol. One microgram of each RNA sample was treated with RNAse-free DNAse (Promega, Leiden, The Netherlands) to avoid DNA contamination and further reverse transcribed (iScript cDNA Synthesis Kit, Bio-Rad

Laboratories, Nazareth Eke, Belgium). Quantitative real-time PCR (qPCR) was performed in the iQ5 Monocolor Real Time PCR detection system (Bio-Rad Laboratories) using 2 ng of cDNA from each sample, 0.375 µM of specific primers (Invitrogen, Merelbeke, Belgium) and SYBR Green (Bio-Rad Laboratories) in a total volume of 25 µL. Sequences of specific primers for each gene are listed in table II.

The amplification protocol was conducted as follows: 30 sec denaturation at 95°C, 45 sec annealing at 60°C, and 15 sec elongation at 79°C for 40 cycles. The fluorescence signal was monitored at the end of each elongation step. For quantitative analyses, amplification was performed in the same conditions for each target gene with serial dilutions of a mix of the cDNA templates. Each sample was normalized to the relative amplification of glyceraldehyde phosphate deshydrogenase (GAPDH). Calculation of cycle threshold values, standard curve preparation, and relative quantification of mRNA in the sample were performed using the post-run data analysis software provided with the iCycler system (Bio-Rad Laboratories).

Spinal cord section preparations and immunohistochemistry

Animals were deeply anaesthetized with xylazine (0.5 mL.kg-1) and ketamine (1.6 mL.kg-1) and killed by transcardiac perfusion with saline solution (0.9% NaCl) followed by cold (4°C) 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Immediately after perfusion, spinal cord was carefully removed from the vertebral column and post-fixed overnight in 4% PFA at 4°C. To cryopreserve the tissues, spinal cords were further incubated in 30% sucrose dissolved in PBS at 4°C for 72 h. Horizontal sections from lumbar enlargements (30 µm) were cut using a sliding microtome and stored in a cryoprotective solution at -20°C until use. For immunohistochemistry, randomly selected sections from each animal were washed twice with Tris-buffered saline (TBS) pH 7.4 for 10 min at 22°C, mounted on Superfrost® glass slides (Menzel-Gläser, VWR, Leuven, Belgium) and dried out overnight. Sections were then permeabilized in TBS containing 0.25% Triton X-100 (Fluka,

Sigma-Aldrich NV/SA, Bornem, Belgium) for 10 min and blocked in TBS containing 10% skimmed milk and 0.25% Triton X-100 for 30 min to avoid non specific labeling. For immunostaining, sections were incubated overnight at 4°C in blocking solution (1% skimmed, 0.25% Triton X-100 dissolved in TBS) with the following primary antibodies: rabbit polyclonal anti-PICK1 (1:1000, Abcam, Cambridge, United-Kingdom), mouse monoclonal anti-glial fibrillary acidic protein (GFAP, 1:400, Sigma-Aldrich NV/SA), mouse monoclonal anti-rat cluster of differentiation molecule 11b (CD11b, 1:500, Chemicon, Millipore, Brussels, Belgium) and chicken polyclonal anti-choline acetyltransferase (ChAT, 1:500, Aves Labs Inc., USA). The specificity of the anti-PICK1 antibody was thoroughly validated in a previous study after reducing the expression of PICK1 using small interfering RNA [27]. Furthermore, the specific PICK1 labeling was herein confirmed by pre-incubating the anti-PICK1 antibody dilutions with the blocking peptide overnight at 4°C under shaking (1:500, Abcam). Simultaneously, an equivalent volume of TBS containing 0.25% Triton X-100 has been added to antibody dilution to work as a positive labeling control. Immunolabeling was revealed using Alexa Fluor conjugated secondary antibodies purchased from Invitrogen as follow: Alexa Fluor 488 goat anti-rabbit (1:500), Alexa Fluor 555 goat anti-mouse (1:500), Alexa Fluor 488 donkey anti-mouse (1:500) and Alexa Fluor 555 goat anti-chicken (1:500) in blocking solution for 1h at 22°C. Labeling of the nuclei was performed at the end of the immunolabeling protocol using DAPI (1:5,000, Sigma-Aldrich) diluted in TBS for 10 min at 22°C. After embedding in Fluoroprep (bioMERIEUX Benelux S.A./N.V, Brussels, Belgium), lumbar spinal cord sections were examined with a fluorescent microscope (total sections) (Evos fl, AMG, Westburg, Leusden, Belgium) or a confocal fluorescent microscope for colocalization (BX61, Olympus Belgium N.V., Aartselaar, Belgium). Images were further analyzed using ImageJ and Automerge Photoshop tool (Adobe Photoshop CS5) has been used to rebuild total spinal cord sections.

Results

PICK1 expression in lumbar spinal cord

Immunodetection of PICK1 in the rat spinal cord revealed a diffuse staining in the dorsal layers of wild-type lumbar spinal cord sections while large round cell bodies were also labeled throughout the grey matter (Fig. 1A). At higher magnification, intensely immunoreactive cells within the ventral horns showed the typical morphology of motor neurons. This corroborates previous work showing that in the spinal cord, PICK1 was essentially expressed in neurons [6]. The specificity of the immunolabeling was validated by pre-absorbing the PICK1 antibody using the synthetic immunization peptide, a procedure that considerably decreased the detected signal.

As in late stage of the disease, hSOD1^{G93A} rats show a total loss of motor neurons, PICK1 expression was first specifically examined in lumbar spinal cord sections from transgenic animals selected for their initial hind-limb paralysis. At end-stage, PICK1 immunostaining appeared homogeneous throughout the grey matter (Fig. 1B). Here again, the specificity of the labeling was confirmed by pre-absorbing the PICK1 antibody with the immunization peptide. Focus on the ventral horn at higher magnification revealed small cells immunostained with the PICK1 antibody. Compared to PICK1 positive cells identified in sections from wild-type animals, the positive cells in sections from transgenic rats showed a totally distinct morphology.

The expression of PICK1 in dorsal and ventral horns was compared by measuring the mean intensity of immunostaining per area. As shown in figure 1C, PICK1 staining was systematically higher in dorsal horns in the lumbar spinal cord of both wild-type and diseased transgenic animals, as compared to their matching ventral horns. This result is in agreement with a previous report studying PICK1 expression in healthy spinal cord samples by Western-blotting [7].

Overall PICK1 expression in the spinal cord is not altered with ALS disease progression

Beyond the progressive loss of motor neurons, a common feature in spinal tissues from ALS patients and related transgenic animals is the massive proliferation of astroglial and microglial cells. To correlate PICK1 expression with disease progression, we performed qPCR in order to quantify the genetic expression of PICK1 as well as ChAT, a specific marker of motor neurons, GFAP, a specific marker of astrocytes and the ionized calcium binding adaptor molecule 1 (Iba1), a specific marker of microglia.

At presymptomatic stage, no difference in ChAT mRNA expression was detected for transgenic animals as compared to wild-type, neither in cervical nor in lumbar spinal cord samples (Fig. 2A). In contrast, both GFAP and Iba1 mRNA levels appeared significantly higher in lumbar spinal cord samples of transgenic animals compared to wild-type whereas cervical expression remained similar in both genotypes (Fig. 2B and C). At this presymptomatic stage, we did not detect any modification of PICK1 expression in the cervical or lumbar spinal cord of transgenic animals as compared to wild-type littermates (Fig. 2D).

As the hind-limb paralysis developed, ChAT mRNA levels dramatically decreased in lumbar and cervical spinal cord samples of diseased animals as compared to wild-type (Fig. 2A). We also observed an enhanced expression of GFAP mRNA in both cervical and lumbar tissues from diseased animals as compared to wild-type (Fig. 2B). At this early stage of the disease, increased expression of Iba1 was only evidenced in lumbar spinal cord samples whereas cervical levels remained unchanged compared to wild-type (Fig. 2C). With respect to PICK1 mRNA, no significant change in expression appeared to be associated with the developing paralysis in either cervical or lumbar spinal cord samples (Fig. 2D).

At the end-stage of the disease (animals showing generalized paralysis), a decreased ChAT expression was evidenced at both cervical and lumbar levels (Fig. 2A). However, such decrease, examined in comparison to samples from wild-type animals, appeared less

pronounced than the considerable loss observed at disease onset. Consistent with the development of massive gliosis, a significant increase in GFAP and Iba1 expressions was evidenced in both cervical and lumbar spinal cord as compared to wild-type (Fig. 2B and C). In lumbar spinal cord samples from hSOD1^{G93A} rats, the expression of PICK1 was not significantly different as compared to samples from wild-type animals. On the contrary, we noticed a significant decreased cervical PICK1 expression in the diseased animals (Fig. 2D).

Astroglial expression of PICK1 in the spinal cord at end-stage of ALS

The preserved global mRNA levels of PICK1 despite the extensive loss of motor neurons and widespread gliosis in the spinal cord of paralyzed hSOD1^{G93A} rats prompted us to further characterize its presence in the different CNS cell types in both wild-type and transgenic animals. To this aim, co-staining experiments for PICK1 and markers of motor neurons, astrocytes and microglia were performed in lumbar sections from end-stage hSOD1^{G93A} rats or age-matched wild-type animals and analyzed by confocal microscopy. In wild-type animals, ChAT-positive cells showed the typical morphology of motor neurons, with a large polygonal soma and a low density nucleus (DAPI staining) (Fig. 3A). These cells showed robust PICK1 labeling, suggesting that PICK1 is predominantly expressed in motor neurons in healthy animals. In samples from diseased transgenic animals, a higher density of ChAT-positive cells was evidenced, of smaller size and stellate profile. PICK1 expression was systematically detected in these ChAT-positive cells.

As a typical marker of astrogliosis, GFAP immunostaining was weakly detected in samples from wild-type animals (Fig. 3B) and no colocalization of PICK1 and GFAP could be evidenced, indicating that PICK1 is not expressed in astrocytes in healthy tissues. In lumbar spinal sections from paralyzed hSOD1^{G93A} animals, intense GFAP immunolabeling evidenced typical astrocytic activation and proliferation with obvious colocalization with PICK1 in lumbar ventral horns, suggesting that hSOD1^{G93A} expression triggered PICK1 expression or

stabilization in activated astrocytes. Of importance all astrocytes showing the typical hypertrophic phenotype and densely stained for GFAP were positive for PICK1.

Finally, CD11b labeling was performed in order to evidence activated microglia (Fig. 3C). In agreement with the resting state of microglia in healthy conditions, CD11b labeling was barely detectable in samples from wild-type animals. In contrast, a strong CD11b labeling was observed in lumbar sections from end-stage hSOD1^{G93A} rats, demonstrating the activation and proliferation of microglial cells associated with disease progression. In contrast to astrocytes, PICK1 did not colocalize with CD11b, indicating that PICK1 is expressed neither in resting nor in activated microglia.

Together, these immunohistochemical investigations revealed that PICK1 systematically colocalized within GFAP as well as ChAT-positive cells in the lumbar spinal cord from ALS end-stage animals. This unexpectedly suggests that ChAT and GFAP are expressed in the same cells in samples from paralyzed animals. Therefore, we examined whether activated astrocytes that stained positive for GFAP in sections from diseased hSOD1^{G93A} rats also expressed the motor neuron marker ChAT. As illustrated in figure 3D, ChAT positive cells in sections from wild-type animal were not stained with the GFAP antibody. In contrast, we evidenced an unambiguous colocalization of ChAT and GFAP stainings in sections from the transgenic animals at end-stage of ALS. All GFAP stained cells showing the typical stellate morphology of reactive phenotype were positive for ChAT indicating that activation of the astrocytes during the progression of the paralysis is accompanied by an induction of ChAT. Of note, some small GFAP positive processes that most likely belong to non-activated cells were not stained for ChAT.

Discussion

Initially characterized for its interaction with PKCα [28], PICK1 is an ubiquitous protein mainly expressed in the CNS and testes [1]. Notwithstanding the apparent lack of major neurological symptoms in PICK1 knockout mice [4], this PDZ protein has been implicated in several neurological disorders [6,29,30]. Indeed, disruption of PICK1 in neurons demonstrated its essential role in the control of synaptic plasticity, regulating the cell surface expression of GluR2-containing AMPARs (functional plasticity) [2] and, more recently, the activity-dependent remodeling of dendritic spines (structural plasticity) [31]. Furthermore, PICK1 interacts with a plethora of partners regulating diverse neurotransmissions in the brain (reviewed in [3]). In pathological conditions, PICK1 also participates in excitotoxic neuronal death, triggering enhanced AMPAR-induced Ca^{2+} influx in damaged hippocampal neurons [8,9].

In this study, we evaluated putative changes in PICK1 expression in a transgenic rat model of ALS. Indeed, PICK1 has been associated with molecular components of ALS pathophysiology, such as the ionotropic glutamate AMPAR subunit GluR2 [22], the glutamate transporter EAAT2b/GLT-1b [21] and the SRR [23]. In lumbar and cervical spinal cord samples of transgenic animals, we did not observe any significant modification of PICK1 genetic expression throughout disease progression as compared to age-matched wild-type littermates. The exception is the down-regulation of PICK1 expression at the late stage of the disease in cervical sections, where micro- and astrogliosis also appeared later during disease progression. Of note, it has been previously described that PICK1 is exclusively expressed in neurons in the spinal cord of rats [6]. Furthermore, motor neurons in mutant hSOD1 mice were reported to express high levels of PICK1 at the presymptomatic stage [32]. Therefore, the preserved PICK1 expression at end-stage appears paradoxical with the severe motor neuron loss in ALS. Colocalization of PICK1 within ChAT-positive cells confirmed that

PICK1 was predominantly expressed by motor neurons in the ventral horns of healthy rats. On the contrary, PICK1 colocalized with all hypertrophic and stellate GFAP-positive cells but not with CD11b-positive cells in ventral horns of the spinal cord in diseased animals, demonstrating the exclusive expression of PICK1 in reactive astrocytes. Altogether, the preservation of spinal cord PICK1 mRNA levels in hSOD1^{G93A} rats likely reflects a dynamic shift of expression, from motor neurons to astrocytes, during disease-associated tissue remodeling.

Another original finding of this study was the evidence for expression of ChAT, a specific marker of motor neurons, in activated astrocytes within the spinal cord of diseased animals. To our knowledge, ChAT labeling in spinal cord samples from animal models of ALS at end-stage has never been explored. However, ChAT expression has been documented in activated astrocytes in the brain of a murine model of Alzheimer's disease [33] as well as in astroglial cultures [34]. Even though this may appear provocative, abnormal glial expression of neuronal markers has been reported in several pathological conditions. Indeed, the neuronal NO synthase is up-regulated in astrocytes, but not in microglia, in the spinal cord of transgenic models of ALS but also in the brain of Parkinson's and Alzheimer's diseases-related models [35-37]. Similarly, expression of the neuron-specific glutamate transporter, EAAC1, is commonly observed in glioma [38]. Therefore, the persistent expression of ChAT mRNA detected by qPCR at disease end-stage, where few or no motor neurons are present, may be related to an induction of this neuronal marker in reactive astrocytes during ALS progression.

In paralyzed hSOD1^{G93A} rats, PICK1 expression was found to be enhanced in reactive astrocytes of the spinal cord. Astrocytic expression of PICK1 has already been described in hippocampal, hypothalamic and cortical astroglial cultures from healthy animals, where PICK1 was associated with SRR and enhanced D-serine production and release [23,39,40].

Mainly expressed in glia, SRR converts L-serine into D-serine, the endogenous co-agonist of NMDAR [41]. Elevated levels of D-serine have been demonstrated in ALS patients and transgenic mice, likely contributing to glutamate-induced toxicity [14]. The importance of D-serine in excitotoxic processes associated with ALS is further supported by data demonstrating a loss of the D-serine metabolizing enzyme, D-amino acid oxidase, in a familial form of the disease [42]. Considering the importance of D-serine as an endogenous modulator of glutamate transmission, inhibition of SRR is of potential interest for neurological diseases where overstimulation of NMDA receptors plays a pathological role [43]. In ALS, such approach should however take into account the herein demonstrated enhanced expression of PICK1 in activated astrocytes and the possible consequence on D-serine synthesis and release.

Beside releasing gliotransmitters, astrocytes actively participate in the control of glutamate homeostasis in healthy CNS through specific uptake and recycling (reviewed in [44]). The dysfunction of the astrocytic high-affinity glutamate transporter EAAT2 in ALS patient or its equivalent GLT-1 in rodent models likely explains the elevated glutamate levels in the CSF that is associated with ALS [15,45]. While the reduced glutamate transport activity in ALS can be correlated with a decreased expression of EAAT2a/GLT-1a, accumulating data provide evidence for an increased expression of the EAAT2b/GLT-1b isoform in both ALS patients and transgenic animal models [16,17]. Such opposite regulation of glutamate transporter splice variants in ALS and the consequences on glutamate handling remain to be understood. However, it is noteworthy that PICK1 was shown to specifically interact with GLT-1b [21]. Indeed, the C-terminal splicing endows GLT-1b with the capacity to interact with PDZ domain proteins, promoting its stability at the cell surface [21,46,47]. Therefore, the increased expression of both GLT-1b and its scaffolding partner PICK1 in ALS-derived astroglia certainly deserves further consideration.

ALS is a non-cell autonomous disorder in which vulnerability of both motor neurons and glial cells participates in the pathogenesis of the disease [48]. Indeed, cell-specific expression of mutant SOD1 solely in motor neurons, microglia or astrocytes failed to reproduce ALS in conditional transgenic animals. We here demonstrate that PICK1 is expressed in spinal astrocytes at end-stage of the disease, but not in microglia. As PICK1 is exclusively expressed in neurons in the spinal cord of wild-type animals, our results are consistent with a cellular shift of PICK1 expression from motor neurons to astroglia during the progression of ALS. Release of the gliotransmitter D-serine and the efficient clearance of glutamate are both essential astrocyte activities in the healthy CNS. It is remarkable that increased release of D-serine and impaired glutamate transport are among the proposed mechanisms underlying glutamate-mediated excitoxicity in ALS. As interactions of both SRR and the glutamate transporter isoform GLT-1b with the PDZ protein PICK1 have been described, further studies should determine the functional involvement of astrocytic PICK1 in ALS.

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Author Contributions

MCF was responsible for executing the entire research project, the statistical analyses and writing the manuscript. SG, CB and AD assisted technically for rat breeding, animal sacrifices and RT-qPCR. EH directed the experiments and analyses and the writing of the manuscript. All authors read and approved the final manuscript.

References

1. Xia J, Zhang X, Staudinger J, Huganir RL. Clustering of AMPA receptors by the synaptic PDZ domain-containing protein PICK1. *Neuron* 1999; **22**: 179-187

2. Hanley JG. Molecular mechanisms for regulation of AMPAR trafficking by PICK1. *Biochem Soc Trans* 2006; **34**: 931-935

3. Xu J, Xia J. Structure and function of PICK1. *Neurosignals* 2006; **15**: 190-201

4. Gardner SM, Takamiya K, Xia J, Suh JG, Johnson R, Yu S, Huganir RL. Calcium-permeable AMPA receptor plasticity is mediated by subunit-specific interactions with PICK1 and NSF. *Neuron* 2005; **45**: 903-915

5. Bellone C, Luscher C. Cocaine triggered AMPA receptor redistribution is reversed in vivo by mGluR-dependent long-term depression. *Nat Neurosci* 2006; **9**: 636-641

6. Wang W, Petralia RS, Takamiya K, Xia J, Li YQ, Huganir RL, Tao YX, Yaster M. Preserved acute pain and impaired neuropathic pain in mice lacking protein interacting with C Kinase 1. *Mol Pain* 2011; **7**: 11

7. Atianjoh FE, Yaster M, Zhao X, Takamiya K, Xia J, Gauda EB, Huganir RL, Tao YX. Spinal cord protein interacting with C kinase 1 is required for the maintenance of complete Freund's adjuvant-induced inflammatory pain but not for incision-induced post-operative pain. *Pain* 2010; **151**: 226-234

8. Bell JD, Park E, Ai J, Baker AJ. PICK1-mediated GluR2 endocytosis contributes to cellular injury after neuronal trauma. *Cell Death Differ* 2009; **16**: 1665-1680

9. Dixon RM, Mellor JR, Hanley JG. PICK1-mediated glutamate receptor subunit 2 (GluR2) trafficking contributes to cell death in oxygen/glucose-deprived hippocampal neurons. *J Biol Chem* 2009; **284**: 14230-14235

10. Rothstein JD. Current hypotheses for the underlying biology of amyotrophic lateral sclerosis. *Ann Neurol* 2009; **65 Suppl 1**: S3-S9

11. Van Den Bosch L, Van Damme P, Bogaert E, Robberecht W. The role of excitotoxicity in the pathogenesis of amyotrophic lateral sclerosis. *Biochim Biophys Acta* 2006; **1762**: 1068-1082

12. Rothstein JD, Tsai G, Kuncl RW, Clawson L, Cornblath DR, Drachman DB, Pestronk A, Stauch BL, Coyle JT. Abnormal excitatory amino acid metabolism in amyotrophic lateral sclerosis. *Ann Neurol* 1990; **28**: 18-25

13. Takuma H, Kwak S, Yoshizawa T, Kanazawa I. Reduction of GluR2 RNA editing, a molecular change that increases calcium influx through AMPA receptors, selective in the spinal ventral gray of patients with amyotrophic lateral sclerosis. *Ann Neurol* 1999; **46**: 806-815

14. Sasabe J, Chiba T, Yamada M, Okamoto K, Nishimoto I, Matsuoka M, Aiso S. D-serine is a key determinant of glutamate toxicity in amyotrophic lateral sclerosis. *EMBO J* 2007; **26**: 4149-4159

15. Rothstein JD, Van Kammen M, Levey AI, Martin LJ, Kuncl RW. Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis. *Ann Neurol* 1995; **38**: 73-84

16. Maragakis NJ, Dykes-Hoberg M, Rothstein JD. Altered expression of the glutamate transporter EAAT2b in neurological disease. *Ann Neurol* 2004; **55**: 469-477

17. Goursaud S, Maloteaux JM, Hermans E. Distinct expression and regulation of the glutamate transporter isoforms GLT-1a and GLT-1b in cultured astrocytes from a rat model of amyotrophic lateral sclerosis (hSOD1G93A). *Neurochem Int* 2009; **55**: 28-34

18. Shaw PJ, Williams TL, Slade JY, Eggett CJ, Ince PG. Low expression of GluR2 AMPA receptor subunit protein by human motor neurons. *Neuroreport* 1999; **10**: 261-265

19. Sun H, Kawahara Y, Ito K, Kanazawa I, Kwak S. Expression profile of AMPA receptor subunit mRNA in single adult rat brain and spinal cord neurons in situ. *Neurosci Res* 2005; **52**: 228-234

20. Van Damme P, Van Den BL, Van Houtte E, Callewaert G, Robberecht W. GluR2-dependent properties of AMPA receptors determine the selective vulnerability of motor neurons to excitotoxicity. *J Neurophysiol* 2002; **88**: 1279-1287

21. Bassan M, Liu H, Madsen KL, Armsen W, Zhou J, Desilva T, Chen W, Paradise A, Brasch MA, Staudinger J, Gether U, Irwin N, Rosenberg PA. Interaction between the glutamate transporter GLT1b and the synaptic PDZ domain protein PICK1. *Eur J Neurosci* 2008; **27**: 66-82

22. Dev KK, Nishimune A, Henley JM, Nakanishi S. The protein kinase C alpha binding protein PICK1 interacts with short but not long form alternative splice variants of AMPA receptor subunits. *Neuropharmacology* 1999; **38**: 635-644

23. Fujii K, Maeda K, Hikida T, Mustafa AK, Balkissoon R, Xia J, Yamada T, Ozeki Y, Kawahara R, Okawa M, Huganir RL, Ujike H, Snyder SH, Sawa A. Serine racemase binds to PICK1: potential relevance to schizophrenia. *Mol Psychiatry* 2006; **11**: 150-157

24. Vermeiren C, Hemptinne I, Vanhoutte N, Tilleux S, Maloteaux JM, Hermans E. Loss of metabotropic glutamate receptor-mediated regulation of glutamate transport in chemically activated astrocytes in a rat model of amyotrophic lateral sclerosis. *J Neurochem* 2006; **96**: 719-731

25. Matsumoto A, Okada Y, Nakamichi M, Nakamura M, Toyama Y, Sobue G, Nagai M, Aoki M, Itoyama Y, Okano H. Disease progression of human SOD1 (G93A) transgenic ALS model rats. *J Neurosci Res* 2006; **83**: 119-133

26. Goursaud S, Focant MC, Berger J, Nizet Y, Maloteaux JM, Hermans E. The VPAC2 agonist peptide histidine isoleucine (PHI) up-regulates glutamate transport in the corpus callosum of a rat model of amyotrophic lateral sclerosis (hSOD1G93A) by inhibiting caspase-3 mediated inactivation of GLT-1a. *FASEB J* 2011; **25**: 3674-3686

27. Haglerød C, Kapik A, Boulland JL, Hussain S, Holen T, Skare O, Laake P, Ottersen OP, Haug FM, Davanger S. Protein interacting with C kinase 1 (PICK1) and GluR2 are associated with presynaptic plasma membrane and vesicles in hippocampal excitatory synapses. *Neuroscience* 2009; **158**: 242-252

28. Staudinger J, Zhou J, Burgess R, Elledge SJ, Olson EN. PICK1: a perinuclear binding protein and substrate for protein kinase C isolated by the yeast two-hybrid system. *J Cell Biol* 1995; **128**: 263-271

29. Dev KK, Henley JM. The schizophrenic faces of PICK1. *Trends Pharmacol Sci* 2006; **27**: 574-579

30. Bertaso F, Zhang C, Scheschonka A, de Bock F, Fontanaud P, Marin P, Huganir RL, Betz H, Bockaert J, Fagni L, Lerner-Natoli M. PICK1 uncoupling from mGluR7a causes absence-like seizures. *Nat Neurosci* 2008; **11**: 940-948

31. Nakamura Y, Wood CL, Patton AP, Jaafari N, Henley JM, Mellor JR, Hanley JG. PICK1 inhibition of the Arp2/3 complex controls dendritic spine size and synaptic plasticity. *EMBO J* 2011; **30**: 719-730

32. Veglianese P, Caron I, Pozzi B, Bendotti C. Changes of AMPA receptors subunits and their associated trafficking proteins (ABP, NSF and PICK-1) in the spinal cord of a mouse model of familial amyotrophic lateral sclerosis. *FENS Forum Abstracts* 2006; **vol. 3**:

33. Nagele RG, D'Andrea MR, Lee H, Venkataraman V, Wang HY. Astrocytes accumulate A beta 42 and give rise to astrocytic amyloid plaques in Alzheimer disease brains. *Brain Res* 2003; **971**: 197-209

34. Wessler I, Reinheimer T, Klapproth H, Schneider FJ, Racke K, Hammer R. Mammalian glial cells in culture synthesize acetylcholine. *Naunyn Schmiedebergs Arch Pharmacol* 1997; **356**: 694-697

35. Carbone DL, Morenoy JA, Tjalkens RB. Nuclear factor kappa-B mediates selective induction of neuronal nitric oxide synthase in astrocytes during low-level inflammatory stimulation with MPTP. *Brain Research* 2008; **1217**: 1-9

36. Catania MV, Aronica E, Yankaya B, Troost D. Increased expression of neuronal nitric oxide synthase spliced variants in reactive astrocytes of amyotrophic lateral sclerosis human spinal cord. *Journal of Neuroscience* 2001; **21**:

37. Simic G, Lucassen PJ, Krsnik Z, Kruslin B, Kostovic I, Winblad B, Bogdanovic N. nNOS expression in reactive astrocytes correlates with increased cell death related DNA damage in the hippocampus and entorhinal cortex in Alzheimer's disease. *Experimental Neurology* 2000; **165**: 12-26

38. Palos TP, Ramachandran B, Boado R, Howard BD. Rat C6 and human astrocytic tumor cells express a neuronal type of glutamate transporter. *Brain Res Mol Brain Res* 1996; **37**: 297-303

39. Dziedzic B, Prevot V, Lomniczi A, Jung H, Cornea A, Ojeda SR. Neuron-to-glia signaling mediated by excitatory amino acid receptors regulates ErbB receptor function in astroglial cells of the neuroendocrine brain. *J Neurosci* 2003; **23**: 915-926

40. Zhuang Z, Yang B, Theus MH, Sick JT, Bethea JR, Sick TJ, Liebl DJ. EphrinBs regulate D-serine synthesis and release in astrocytes. *J Neurosci* 2010; **30**: 16015-16024

41. Wolosker H, Blackshaw S, Snyder SH. Serine racemase: a glial enzyme synthesizing D-serine to regulate glutamate-N-methyl-D-aspartate neurotransmission. *Proc Natl Acad Sci U S A* 1999; **96**: 13409-13414

42. Mitchell J, Paul P, Chen HJ, Morris A, Payling M, Falchi M, Habgood J, Panoutsou S, Winkler S, Tisato V, Hajitou A, Smith B, Vance C, Shaw C, Mazarakis ND, de Belleroche J. Familial amyotrophic lateral sclerosis is associated with a mutation in D-amino acid oxidase. *Proc Natl Acad Sci U S A* 2010; **107**: 7556-7561

43. Oliet SH, Mothet JP. Regulation of N-methyl-D-aspartate receptors by astrocytic D-serine. *Neuroscience* 2009; **158**: 275-283

44. Danbolt NC. Glutamate uptake. *Prog Neurobiol* 2001; **65**: 1-105

45. Howland DS, Liu J, She Y, Goad B, Maragakis NJ, Kim B, Erickson J, Kulik J, DeVito L, Psaltis G, DeGennaro LJ, Cleveland DW, Rothstein JD. Focal loss of the glutamate transporter EAAT2 in a transgenic rat model of SOD1 mutant-mediated amyotrophic lateral sclerosis (ALS). *Proc Natl Acad Sci U S A* 2002; **99**: 1604-1609

46. Gonzalez-Gonzalez IM, Garcia-Tardon N, Cubelos B, Gimenez C, Zafra F. The glutamate transporter GLT1b interacts with the scaffold protein PSD-95. *J Neurochem* 2008; **105**: 1834-1848

47. Gonzalez-Gonzalez IM, Garcia-Tardon N, Gimenez C, Zafra F. Splice variants of the glutamate transporter GLT1 form hetero-oligomers that interact with PSD-95 and NMDA receptors. *J Neurochem* 2009; **110**: 264-274

48. Ilieva H, Polymenidou M, Cleveland DW. Non-cell autonomous toxicity in neurodegenerative disorders: ALS and beyond. *J Cell Biol* 2009; **187**: 761-772

Figure legends

Figure 1:

PICK1 expression in the lumbar spinal cord of wild-type and end-stage hSOD1^{G93A} rats

Lumbar spinal cord sections from wild-type (WT) (A) and hSOD1^{G93A} animals (B) were labeled with rabbit anti-PICK1 antibodies. Pre-absorbing anti-PICK1 antibodies with synthetic immunization peptide dramatically reduced immunolabeling in both wild-type (A') and hSOD1^{G93A} (B'). Focus on the ventral horns showed distinct cell-types stained with PICK1 antibodies between WT (A'') and transgenic spinal animal at latter stage (B''). Shown are typical experiments and surrounded areas with dotted lines indicate ventral horns. Scale bars = 200 µm. (C) Quantification of immunofluorescence signals obtained with anti-PICK1 antibody on lumbar spinal cord sections from WT (left) and hSOD1^{G93A} (right) rats. PICK1 levels in ventral horns were measured as relative to total fluorescent intensities per area in spinal dorsal horns of sections for both genotypes (triplicate for each genotype, n=3) and were compared using the Wilcoxon matched-pairs signed rank test (**p<0.01, *p<0.05)

Figure 2

PICK1 total mRNA expression in cervical and lumbar spinal cord is not altered during ALS.

ChAT (A), GFAP (B), Iba1 (C) and PICK1 (D) relative mRNA expression was measured in samples of cervical and lumbar spinal cords from hSOD1^{G93A} transgenic rats at presymptomatic (left panels), symptomatic (middle panels) and end-stage (right panels) and in their age-matched wild-type littermates. Data shown are mean with SEM from 3 animals in each genotype performed in duplicate and were compared using the Student's t-test. (*p<0.05, **p<0.01, ***p<0.001).

Figure 3

PICK1 is expressed in astrocytes in the lumbar ventral horns of end-stage hSOD1^{G93A} rats

Lumbar spinal cords from wild-type (WT) and end-stage transgenic animals (hSOD1^{G93A}) were co-immunolabeled for PICK1 (in green), ChAT (A), GFAP (B) or CD-11b (C) (in red) and DAPI (in blue). In panel D, ChAT (red) and GFAP (green) were immunolabeled on wild-type and end-stage hSOD1^{G93A} lumbar spinal cords. Colocalization was examined on ventral horns of each section by confocal microscopy. Shown are from a typical experiment performed on at least 3 sections from 3 different animals. Scale bars = 20 µm.

Table I

PICK1 protein partners potentially involved in ALS

Partners	**PICK1 interaction**	**Implication in ALS**
AMPA Receptor	GluR2 subunit trafficking (in PICK1 KO mice, ↘ synaptic plasticity) (Gardner et al., 2005; Hanley 2006)	Ca^{2+} permeability of GluR2 subunit (defect in mRNA Q→R editing process in sALS) (Gardner et al., 2005; Kawahara et al., 2004; Kawahara et al., 2006)
Glutamate transporter GLT-1	GLT-1b isoform (Bassan et al., 2008)	↘ Transport activity in patients & animal models (w/o change in affinity) (Howland et al., 2002; Rothstein et al., 1995) ↘ GLT-1a & ↗ GLT-1b expression in patients (Maragakis et al., 2004)
Serine-racemase	↗ D-serine production (Zhuang et al., 2010)	↗ D-serine in sALS & fALS (Sasabe et al., 2007) NMDAR antagonist (memantine) prolonges survival in ALS mice (Wang and Zhang 2005)
Nicotinic Acetylcholine Receptor (nAChR)	↘ Surface expression of α7 subunit containing proteins (Baer et al., 2007)	α7 subunit is involved in neuroprotection (Akaike et al., 2010)
Dopamine transporter (DAT)	DAT trafficking (Torres et al., 2001)	↘ DAT in in peripheral blood mononuclear cells of ALS patients (Buttarelli et al., 2006) ↘ [Dopamine] in ALS transgenic mice midbrain (Kostic et al., 1997)

Table II :

Sequences of primers used for qPCR

Gene	Forward	Reverse
ChAT	AGCCCTGCTGTGATCTTTGCTCG	CCTTGGCCCAGTCAGTGGGAA
GAPDH	GTCTCCTGTGACTTCAACAG	AGTTGTCATTGAGAGCAATGC
GFAP	TGGCCACCAGTAACATGCAA	CAGTTGGCGGCGATAGTCAT
Iba1	CAGAATGATGCTGGGCAAG	CCTCCAATTAGGGCAACTCA
PICK1	AAGGTGAAGGAGATGGACGA	GCAGGATAAGGCGGTACTCA

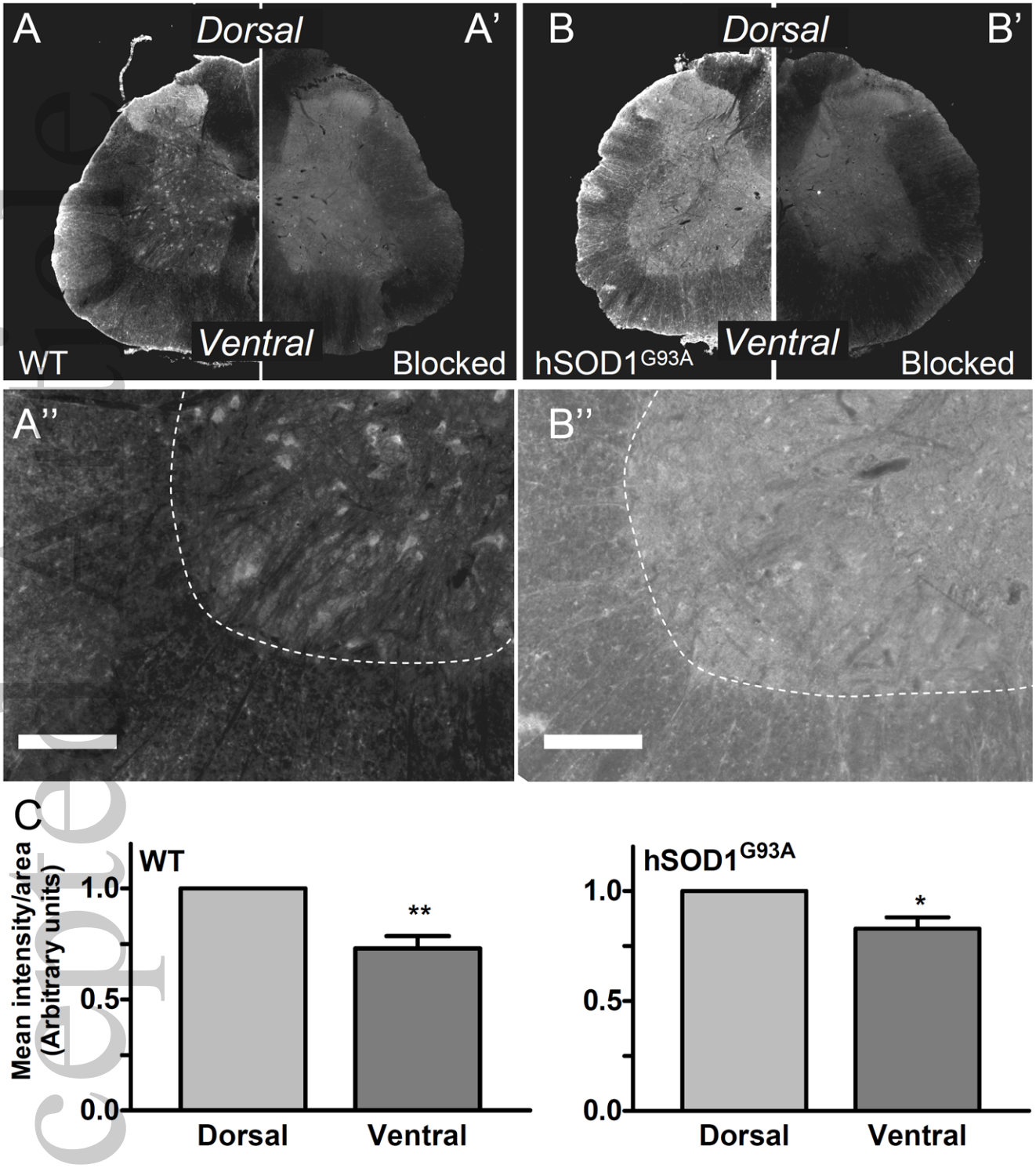

Figure 1

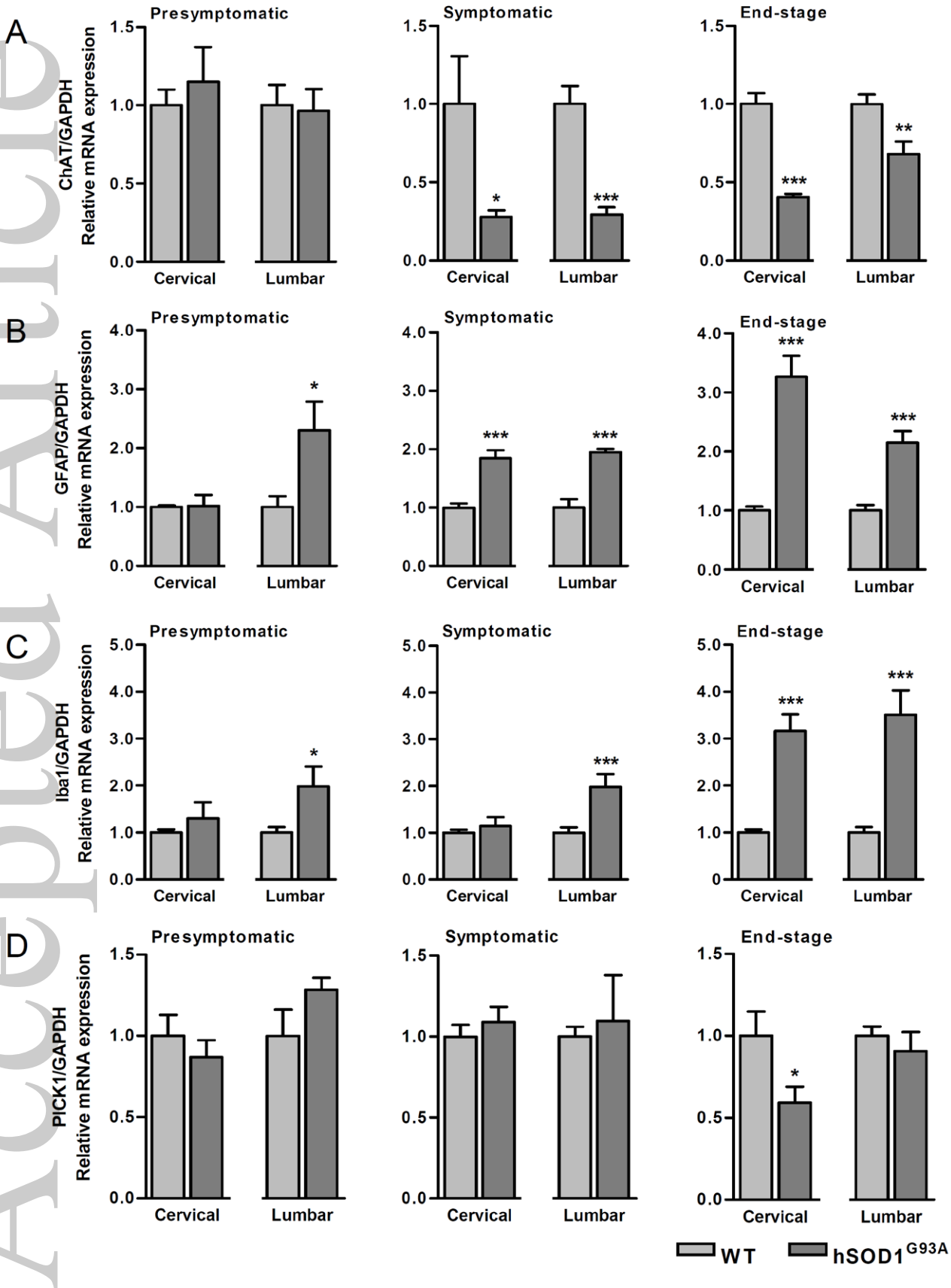

Figure 2

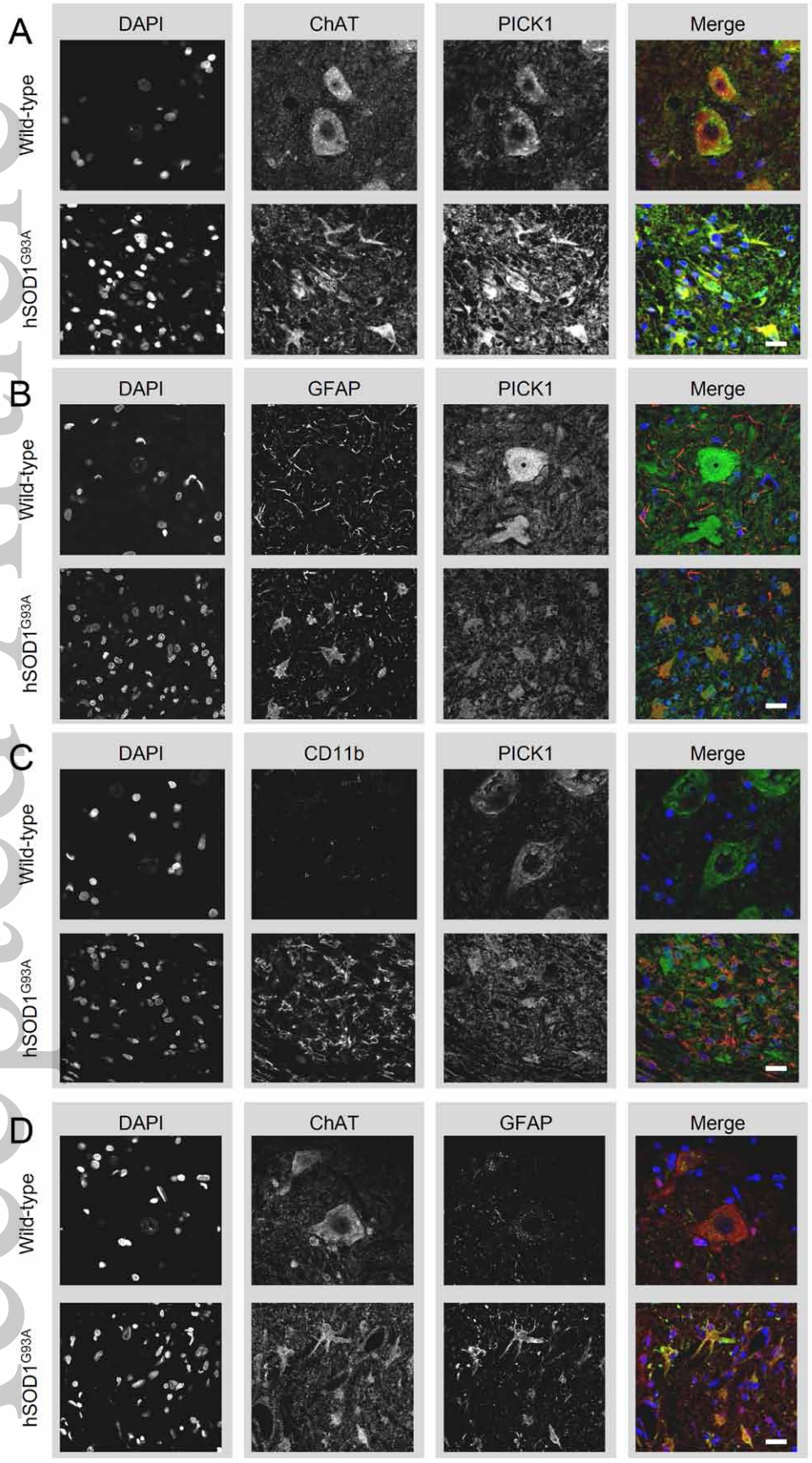

Figure 3