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S100A6, a calcium- and zinc-binding protein, is overexpressed in SOD1 mutant mice, a model for amyotrophic lateral sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterised by selective degeneration of motoneurones. Familial ALS is an age-dependent autosomal dominant disorder in which mutations in the homodimeric enzyme Cu/Zn superoxide dismutase 1 (SOD1) is linked to the disease. An animal model for this disease is a transgenic mouse expressing the mutated human $SOD1^{G93A}$ gene. Recent electrophysiological data emphasised that the striking selective vulnerability of motoneurones might be due to their differential calcium buffering capacities. Therefore we have investigated, using immunohistochemistry, the expression of different calcium binding proteins in brainstem and spinal cord from normal and SOD1 mutated mice. Among the 13 calcium-binding proteins screened, only one, S100A6, a homodimeric calcium-binding protein able to bind four Zn^{2+}, appeared to be highly expressed in the SOD1 mutated mice. In brainstem, reactive astrocytes, but not motoneurones, from several regions, including nerve 12 root, were highly S100A6-positive. Hypoglossal nucleus was negative for S100A6. In dorsal root, reactive astrocytes from both white matter and anterior horn were highly reactive. If overexpression of S100A6 is specific for ALS, it will be a valuable diagnostic marker for this disease.

Keywords: Amyotrophic lateral sclerosis; Calcium binding protein; Motoneuron; Neurodegeneration; Reactive astrocyte; S100A6

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterised by selective motoneurone degeneration in brainstem, spinal cord, and motor cortex [1]. Symptoms of ALS are muscle atrophy, weakness and fasciculation. Besides motoneurone degeneration, atrophy of ventral roots, corticospinal tract degeneration, somatic and axonal inclusions of aberrant neurofilament proteins and astrogliosis occurred. The incidence of ALS is approximately 1/10 000. In familial ALS cases, 20% are linked to approximately 40 different identified mutations in the Cu/Zn superoxide dismutase (SOD1) gene [2,3]. To investigate structural changes on the molecular level, transgenic mice model carrying an additional copy of the mutant human SOD1

Abbreviations: MdV, medullary reticular nucleus, ventral part; 12n, root of hypoglossal nerve; IRt, intermediate reticular nucleus; mlf, medial longitudinal fasciculus; ts, tectospinal tract; ml, lemniscus; ROb, raphe obscurus nucleus

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gene were established. These mice develop similar pathology of motoneurones that resembles human motoneurone disease [3–5]. In the present study, we used the mouse model TgN SOD1^{G93A} introduced by Gurney et al. [6] to investigate the molecular basis of the selective vulnerability of motoneurones. Transgenic mice express high levels of human SOD1 containing a substitution of glycine to alanine at position 93, a change that has little effect on enzyme activity but caused motoneurone disease [7]. In this case, a 'gain of function' mechanism of the mutant SOD1-gene is thought to contribute to the pathogenesis of motoneurone degeneration. In agreement with this model, mice became paralysed in one or more limbs as a result of motoneurone loss from the spinal cord and died by 5 to 6 months of age.

Calcium signalling plays a major role as a messenger for different signal cascades including apoptosis and neuronal cell death. During normal physiological activity, motoneurones show substantial calcium elevations in somatic and dendritic compartments [8,9]. Peak elevations in $[Ca]_i$ were found to reach the micromolar concentration domain during bursts of 10–15 action potentials [10,11]. Investigations using patch-clamp and microfluorimetric measurements indicated that brainstem motoneurones in comparison to other neurones have a low capacity to buffer calcium in the cell soma most likely as a result of low calcium-binding protein expression [12,13]. This specialisation of brainstem motoneurones makes them particularly sensitive to disturbed calcium homeostasis which may explain their vulnerability.

In this work we investigated the differential expression of 13 calcium-binding proteins in normal and mutated mice. We discovered that one of them, S100A6 (calcyclin), was overexpressed in reactive astrocytes from the mutated mice.

The zinc- and calcium-binding protein S100A6 has a molecular mass of 10 kDa and two distinct EF-hand domains that bind Ca^{2+} [14]. S100A6, in vitro, interacts with target proteins such as tropomyosin [15], annexins II and XI [16,17] and caldesmon [18]. S100A6 has a calcium-dependent dual localisation at nuclear envelope and plasma membrane as well as an increase of translocation into a network structure around the nucleus [19,20]. S100A6 is also overexpressed in tumours like acute myeloid leukaemia [21], in neuroblastoma and in melanoma cell lines. S100A6 is expressed in astrocyte subpopulations in rat [22] and in human [23].

Although ALS is known as a motoneurone disease, astrocytes are important actors in the pathology. In general, CNS responds to any neuronal injuries by an increase in number and size of astrocytes expressing GFAP (so-called reactive astrocytes) [24,25]. In humans suffering from ALS, astrogliosis was seen in the cerebral cortex [26] and in cervical and lumbar levels of spinal cord [27]. In ALS, glutamate transfer is impaired. Astrocytes play an important role for such transport and maintain glutamate in the synaptic cleft at low levels. In this work we confirm astrogliosis in the ALS animal model and further show that a zinc/calcium-binding protein (S100A6) is overexpressed in astrocytes.

2. Materials and methods

2.1. Animals

For our investigations we used transgenic animals (purchased from the Jackson Laboratory, ME, USA) heavily suffering from motor dysfunction and normal animals of equal age as control. Animal experiments were carried out in accordance with the guidelines of the Ethics Committee of the Medical Faculty of the University of Göttingen. Transgenic male mice were obtained either from strain B6SJL-TgN (SOD1^{G93A}) 1 Gur or B6SJL-TgN (SOD1G93A) 1 Gur dl. Males were mated with normal female mice (strain: B6SJLF1) to get heterozygote animals for investigation. The strain indicated as dl had a reduced copy of the mutated human superoxide dismutase gene and therefore showed a delayed onset of the ALS phenotype. In this strain, first signs of the disease appear after 246 days ($\pm$32 days). Progression of the disease is then very rapid and after approximately 2 weeks animals were killed because of their disability of ingestion.

2.2. Identification of transgenic animals using diagnostic polymerase chain reaction (PCR)

Transgenic animals were identified using a diagnostic PCR method. After approximately 246 days of life, motor dysfunction was monitored and a piece

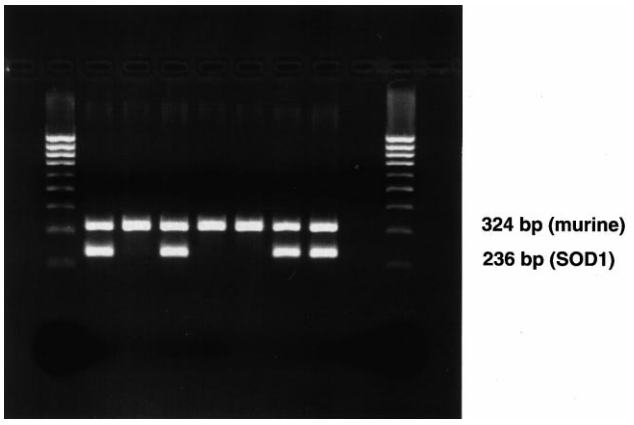

Fig. 1. PCR gel. Lanes 1 and 10, molecular mass marker (bp size: 80, 100, 200, 300, 400, 500, 600, 700, 800, 900, 1031); lanes 2, 4, 7, 8: transgenic mice; lanes 3, 5, 6: normal mice; lane 9, control (primers without DNA).

of the mouse tail was dissected, resuspended in diagnostic buffer (20 mM Tris–HCl (pH 8.4), 50 mM KCl, 0.45% NP40, 0.45% Tween-20) and homogenised. Proteinase K was added for at least 2 h at 55°C. Afterwards the homogenate was heated for 10 min at 95°C and centrifuged at $1000 \times g$ for 1 min. One μl from the supernatant was used for PCR.

Special primers (oIMR113 5′-CAT CAG CCC TAA TCC ATC TGA-3′ and oIMR114 5′-GTA GGT GGA AAT TCT AGT ATC ATC C-3′) were used to amplify a 236 bp product from exon 4 of the human SOD1 gene within the transgene construct. To verify that the PCR performed on normal animal was also successful, specific primers (oIMR042: 5′-CTA GGC CAC AGA ATT GAA AGA TCT-3′ and oIMR043 5′-GTA GGT GGA AAT TCT AGC ATC ATC C-3′) were used amplifying a 324 bp product from the endogenous Il-2 gene. Mastermix (Quiagen, Hilden, Germany), primers, ultraclean water and DNA were mixed. PCR was performed using 94°C 4 min for initial denaturation, 35 cycles (92°C for 1 min/60°C for 1 min/72°C for 1 min) and a final elongation of 5 min at 72°C. Ten μl of each amplification product was run on a 2% agarose gel in parallel with a molecular mass marker (100 bp ladder, MBI Fermentas, Vilnus, Lithuania), and stained with ethidium bromide (Fig. 1).

2.3. Fixation of tissue

Mice were anaesthetised and transcardially perfused using 100 mM phosphate-buffered saline (PBS) (pH 7.4) for 2 min to wash out the blood, followed by a solution of 4% buffered paraformaldehyde in 0.2 M phosphate buffer (pH 7.4) for at least 15 min. Spinal cord and brainstem were dissected and post-fixed for at least 4 h with PAF 4%. They were rinsed for 1 day in 0.1 M PBS (pH 7.4) and dehydrated in alcohol 70° (24 h), 90° (24 h) and 100° (24 h). Afterwards they were immersed in toluol (16 h) and included in paraffin. Five-μm sections were used for immunohistochemistry.

2.4. Immunohistochemistry

Specific polyclonal antibody raised in goat against human recombinant S100A6 was previously characterised [29]. Brain sections were deparaffinated and rehydrated in alcohol (100° and 90°). Endogenous peroxidase activities were minimised by incubation in a solution of methanol with 1% H_2O_2 at room temperature for 30 min. Slides were rinsed under continue flow of water during 10 min, in distilled water and in 50 mM Tris-buffered saline (TBS) (pH 7.4). Non-specific labelling of the secondary antibody was blocked by incubating the sections at 37°C with 5% horse normal serum (Vector Laboratories) for 30 min. The primary antibody was incubated at 4°C overnight. The S100A6 antibody was diluted at 1/10 000 in a TBS solution with bovine serum albumin (3%). Slides were incubated with the biotinylated anti-goat antibody (1/100) for 30 min. Staining was done with avidin–biotin–peroxidase complex (ABC) kit reagents (Vector Laboratories) with diaminobenzidine/H_2O_2 as the chromogenic substrates. Some sections were counterstained with cresyl violet for identification of intact cell bodies.

3. Results

Mice carrying an additional mutated human $SOD1^{G93A}$ gene were identified using PCR method with specific primers for SOD1 gene and a murine Il-2 gene as a positive PCR-control (Fig. 1). PCR showed clearly the existence of the Il-2 murine gene in every mouse investigated. As predicted by the mating strategy approximately 50% of the offspring were $SOD1^{G93A}$-positive. The verification was successful

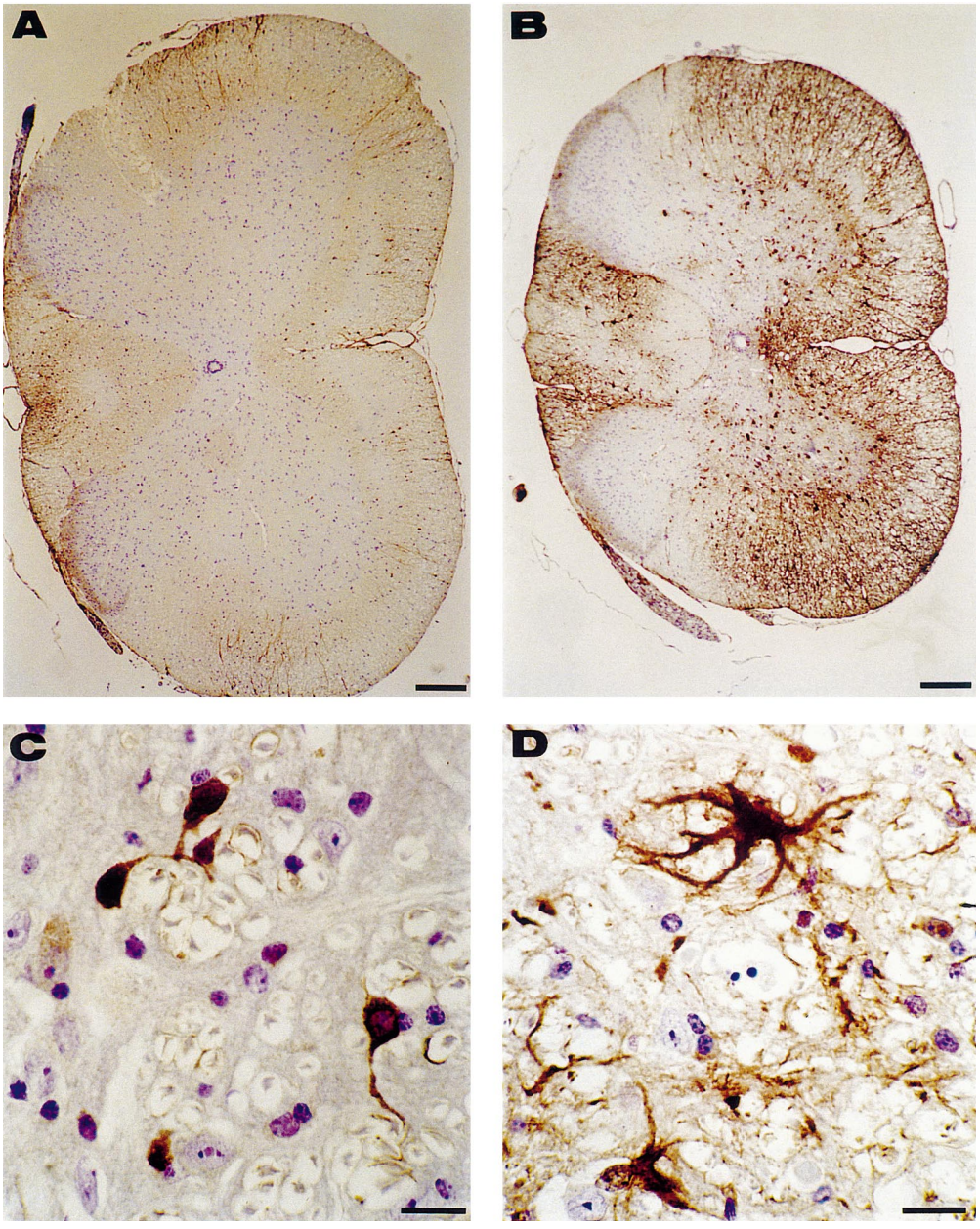

Fig. 2. S100A6 immunohistochemistry of mice spinal cord (A,B) and brainstem (C,D) counterstained with cresyl violet. Normal mice (A,C); SOD1 mutated mice (B,D). S100A6 is restricted to few astrocytes in normal mice whereas S100A6 is overexpressed in both white matters and anterior horns of SOD1 mutated mice (B). Reactive astrocytes are heavily labelled (D). Scale bars: A,B = 200 μm; C,D = 20 μm.

for both very young animals and those which are heavily suffering of motor disorders. We observed no motor disorders in animals tested negative for the mutated human SOD1 ^{G93A} gene. The amplified fragment of the Il-2 murine gene is larger than the fragment for the SOD1 gene and settled first after gel electrophoresis. According to the basepair ladder supplied by Fermentas, the size for the fragments of 346 bp and 236 bp respectively, was confirmed. As a negative control in every PCR experiment one assay lacking mouse DNA was added (Fig. 1, lane 9).

S100A6 immunohistochemistry showed labelling in both normal and SOD1^{G93A}-positive animals. In nor-

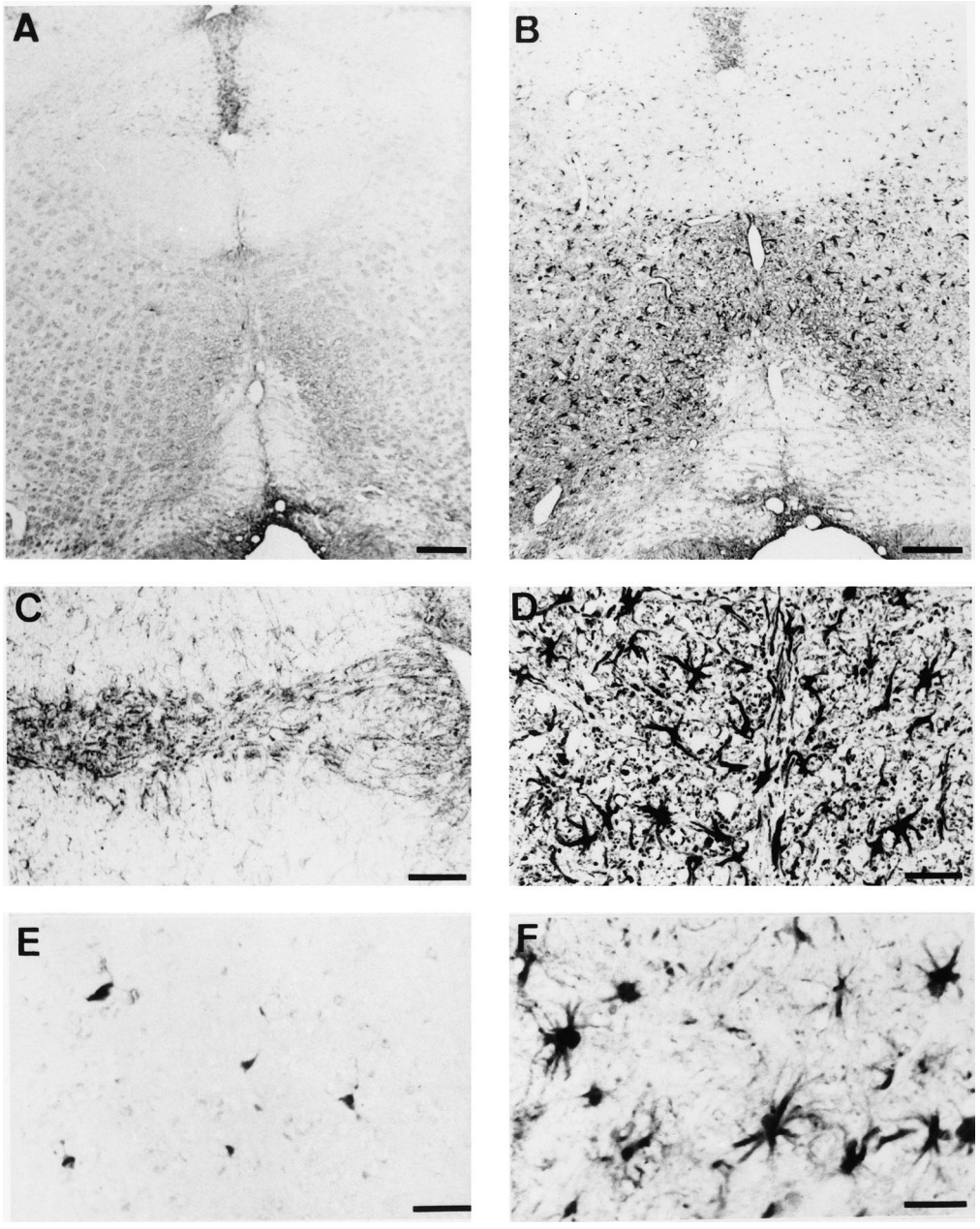

Fig. 3. GFAP (A–D) and S100A6 (E,F) immunohistochemistry of brainstem from normal (A,C,E) and SOD1 mutated (B,D,F) mice. Reactive astrocytes are numerous in SOD1 mutated mice (B,D), positive for GFAP (B,D) and S100A6 (F). Scale bars: A,B = 200 μm; C–E = 60 μm; F = 40 μm.

mal animals, few labelled cells, both in the spinal cord (Fig. 2A) and brainstem (Fig. 2C), could be observed. In spinal cord, positive S100A6 cells that were present in both white matter and grey matter are astrocytes. In $SOD1^{G93A}$-positive animals the labelling was conspicuously more intense compared to normal animals. S100A6 was significantly overexpressed in both white and grey matter of the spinal cord (Fig. 2B) and in some brainstem areas (Fig. 2D). In the grey matter glial cells located in the anterior horn were S100A6-positive (Fig. 2B). Higher magnification clearly showed the typical shape of astrocytes with their bipolar organisation surrounding neurones and vessels (Fig. 2C). In the mutated

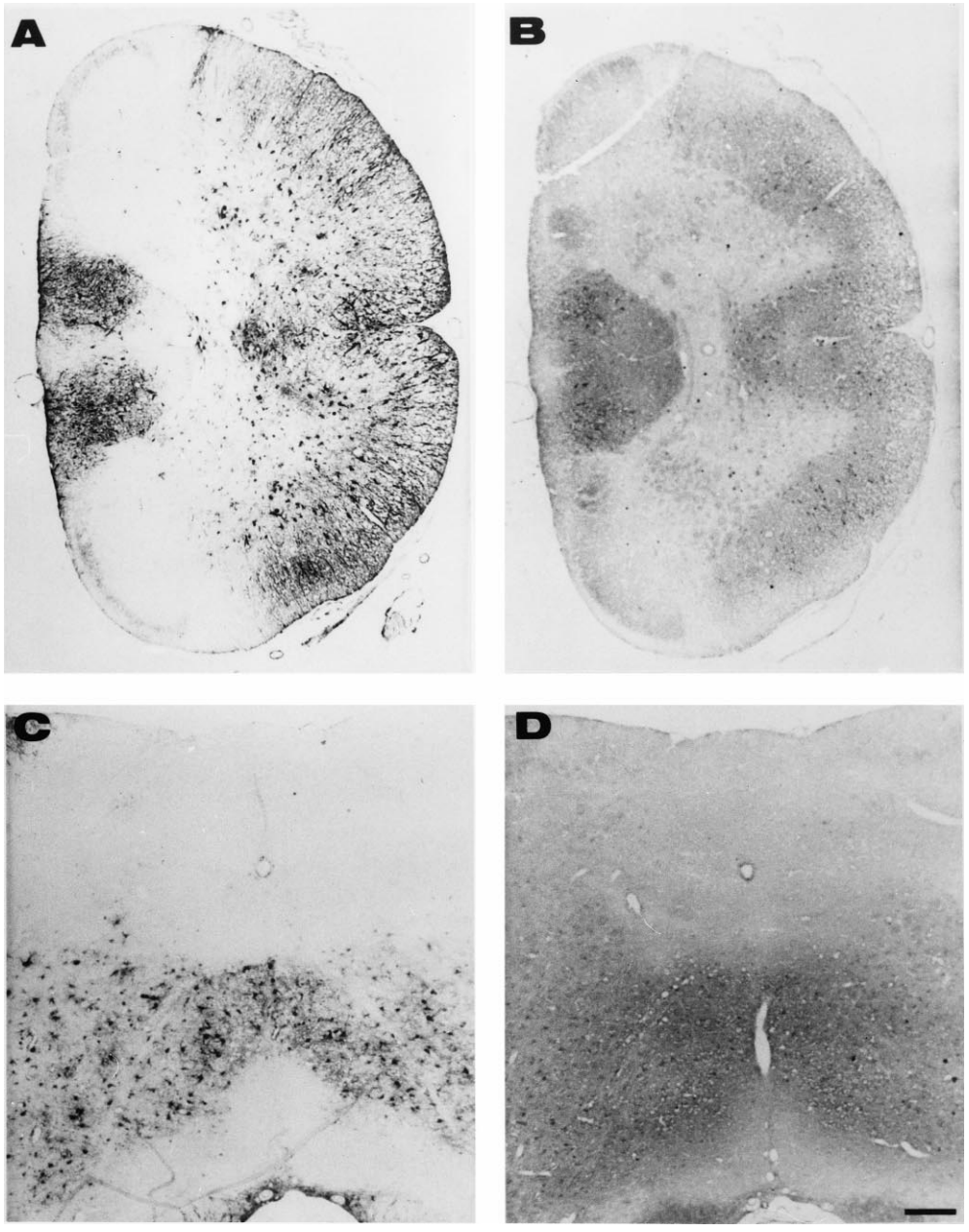

Fig. 4. S100A6 immunohistochemistry of spinal cord (A,B) and brainstem (C,D) from SOD1 mutated mice. Preincubation of S100A6 antibody with pure S100A6 eliminated the labelling (B,D). Scale bar = 200 μm.

mice, S100A6-positive astrocytes had a typical morphology of reactive astrocytes with conspicuous processes (Fig. 2D). Heavy S100A6 immunoreactivity for astrocytes was observed in the following brainstem areas: MdV, 12n, IRt, mlf, ts, ml and ROb. In the hypoglossal nucleus essentially no S100A6-positive labelled astrocytes were detected. To confirm the astrocytic cell type, we used GFAP immunohistochemistry in normal (Fig. 3A,C) and SOD1 mutated mice (Fig. 3B,D). Sections from normal animals showed light labelling whereas SOD1 animals showed astrogliosis with heavy labelling. Specificity of the immunohistochemistry was controlled using the S100A6 antibody (1/10 000) preincubated overnight with pure S100A6 (1 μg/ml). When spinal cord and brainstem sections were incubated with

the mixture (antibody+antigen), no staining could be detected (Fig. 4B,D).

4. Discussion

The exact reason for selective impairment of motoneurones during the neurodegenerative disease ALS is still not well understood. Besides impairments in intracellular protein transport or glutamate exposure (excitotoxicity), many results support the idea that cellular calcium overload is a key component to neuronal impairment [30,31]. Our previous results showing that vulnerable motoneurones, in comparison to surviving motoneurones, have a low calcium buffering capacity [10,12,13], are convergent with such a notion. This led us to investigate different calcium-binding proteins in brainstem and spinal cord and examine if this disease has modified their expression in motoneurones. Surprisingly, our immunohistochemical screening using calbindin, calmodulin, parvalbumin, S100A1, S100A2, S100A3, S100A4, S100A5, S100A6, S100A8, S100A9, S100A12 and S100B antibodies showed that (1) only S100A6 was overexpressed in brainstem and spinal cord of transgenic mice and (2) the modifications occurred in reactive astrocytes and not in motoneurones.

It is known that astrogliosis is observed in a variety of conditions in which neuronal injuries are induced and that astrocytes play a role in mediating neuronal degeneration. More specifically in SOD1 mutated mice, astrogliosis is one of the earliest pathological changes observed [32,33]. In humans, a widespread astrogliosis exists in the subcortical white matter from ALS patients [34], and a close temporal correlation between the onset of neuronal degeneration and the beginning of astrogliosis has been demonstrated [35]. Also, a selective loss of glial glutamate transporter in ALS was described [36]. Recently, Trotti et al. [28] demonstrated that SOD1 mutant selectively inactivated a glial glutamate transporter required to inactivate the synaptic action of glutamate. Astrocytes can also release glutamate in a calcium-dependent manner [37]. The reason for the striking upregulation of S100A6, a protein whose gene belongs to a cluster of 15 S100 genes located on chromosome 1q21, is not clear. The exact in vivo functions of S100 proteins are not yet elucidated but some of them are implicated in the regulation of protein phosphorylation, enzyme activities, Ca^{2+} homeostasis, and the dynamics of microtubules and type III intermediate filaments [38]. Interestingly, S100A6, which forms homodimers, binds four Zn^{2+} and therefore may be an important actor in the development of ALS. Catalytic activities of Cu/Zn for dismutation of the superoxide radical into H_2O_2 or O_2 requires enzyme-bound copper which alternates between reduced (Cu^{1+}) and oxidised (Cu^{2+}) forms during two asymmetric catalysis steps. Loss of zinc diminishes superoxide scavenging and increases tyrosine nitration by SOD [39] and alters the coordination of Cu. Mutated SOD1 have a lower affinity for Zn^{2+} [40] allowing rapid reduction of mutant SOD1 to the Cu^{1+} form. The reduced SOD1 mutant would then run the normal catalytic step backwards, converting oxygen to superoxide and promote intracellular damage. Overexpression of S100A6, a zinc-binding protein, would contribute to the depletion of Zn in astrocytes and in motoneurones and accelerate cell damage.

Whether S100A6 is exclusively upregulated in astrocytes from ALS is not known, but it is potentially an interesting tool as a marker, for ALS in particular, for neurodegenerative diseases in general.

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References

[1] P.J. Shaw, C.J. Eggett, Molecular factor underlying selective vulnerability of motor neurons to neurodegeneration in amyotrophic lateral sclerosis, J. Neurol. 247 (2000) I17–I27.

[2] H.X. Deng, A. Hentati, J.A. Tainer, Z. Iqbal, A. Cayabyab, W.Y. Hung, E.D. Getzoff, P. Hu, B. Herzfeldt, R.P. Roos, Amyotrophic lateral sclerosis and structural defects in Cu, Zn superoxide dismutase, Science 261 (1993) 1047–1051.

[3] D.R. Rosen, T. Siddique, D. Patterson, D.A. Figlewicz, P. Sapp, A. Hentati, D. Donaldson, J. Goto, J.P. O'Regan, H.X. Deng, Mutations in Cu/Zn superoxide dismutase gene are associated with familial amyotrophic lateral sclerosis, Nature 362 (1993) 59–62.

[4] M.E. Ripps, G.W. Huntley, P.R. Hof, J.H. Morrison, J.W. Gordon, Transgenic mice expressing an altered murine superoxide dismutase gene provide an animal model of amyotrophic lateral sclerosis, Proc. Natl. Acad. Sci. USA 92 (1995) 689–693.

[5] P.C. Wong, C.A. Pardo, D.R. Borchelt, M.K. Lee, N.G. Copeland, N.A. Jenkins, S.S. Sisodia, D.W. Cleveland, D.L. Price, An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria, Neuron 14 (1995) 1105–1116.

[6] M.E. Gurney, H. Pu, A.Y. Chiu, M.C. Dal Canto, C.Y. Polchow, D.D. Alexander, J. Caliendo, A. Hentati, Y.W. Kwon, H.X. Deng, Motor neuron degeneration in mice that express a human Cu, Zn superoxide dismutase mutation, Science 264 (1994) 1772–1775.

[7] S.I. Liochev, L.L. Chen, R.A. Hallewell, I. Fridovich, The familial amyotrophic lateral sclerosis-associated amino acid substitutions E100G, G93A, and G93R do not influence the rate of inactivation of copper- and zinc-containing superoxide dismutase by H_2O_2, Arch. Biochem. Biophys. 352 (1998) 237–239.

[8] D. Frermann, B.U. Keller, D.W. Richter, Calcium oscillations in rhythmically active respiratory neurons in the brainstem of the mouse, J. Physiol. (Lond.) 515 (1999) 119–131.

[9] M. Lipps, B.U. Keller, Activity-related calcium dynamics in motoneurons of the nucleus hypoglossus from mouse, J. Neurophysiol. 82 (6) (1999) 2936–2946.

[10] D. Frermann, Intrazelluläre Kalzium – Oszillationen im rhythmisch aktiven respiratorischen Netzwerk der Medulla oblongata der Maus, Ph.D. thesis, University of Göttingen, 1999.

[11] T. Ladewig, B.U. Keller, Simultaneous patch-clamp recording and calcium imaging in a rhythmically active neuronal network in the brainstem slice preparation from mouse, Pflügers Arch. Eur. J. Physiol. 440 (2000) 322–332.

[12] M.B. Lips, B.U. Keller, Endogenous calcium buffering in motoneurons of the nucleus hypoglossus from mouse, J. Physiol. (Lond.) 511 (1998) 105–117.

[13] J. Palecek, M.B. Lips, B.U. Keller, Calcium dynamics and buffering in motoneurons of the mouse spinal cord, J. Physiol. (Lond.) 520 (Pt. 2) (1999) 485–502.

[14] B.W. Schäfer, C.W. Heizmann, The S100 family of EF-hand calcium-binding proteins: functions and pathology, Trends Biochem. Sci. 21 (1996) 134–140.

[15] N.L. Golitsina, J. Kordowska, C.L. Wang, S.S. Lehrer, Ca^{2+}-dependent binding of calcyclin to muscle tropomyosin, Biochem. Biophys. Res. Commun. 18 (1996) 220.

[16] A. Filipek, U. Wojda, W. Lesniak, Interaction of calcyclin and its cyanogen bromide fragments with annexin II and glyceraldehyde-3-phosphate dehydrogenase, Int. J. Biochem. Cell. Biol. 27 (1995) 1123–1131.

[17] T. Sudo, H. Hidaka, Regulation of calcyclin (S100A6) binding by alternative splicing in the N-terminal regulatory domain of annexin XI isoforms, J. Biol. Chem. 273 (1998) 6351–6357.

[18] R.S. Mani, W.D. McCubbin, C.M. Kay, Calcium-dependent regulation of caldesmon by an 11-kDa smooth muscle calcium-binding protein, caltropin, Biochemistry 31 (1992) 11896–11901.

[19] A. Mandinova, D. Atar, B.W. Schäfer, M. Spiess, U. Aebi, C.W. Heizmann, Distinct subcellular localization of calcium binding S100 proteins in human smooth muscle cells and their relocation in response to rises in intracellular calcium, J. Cell Sci. 111 (1998) 2043–2054.

[20] T.B. Stradal, M. Gimona, Ca^{2+}-dependent association of S100A6 (Calcyclin) with the plasma membrane and the nuclear envelope, J. Biol. Chem. 274 (1999) 31593–31596.

[21] B. Calabretta, L. Kaczmarek, W. Mars, D. Ochoa, C.W. Gibson, R.R. Hirschhorn, R. Baserga, Cell-cycle-specific genes differentially expressed in human leukemias, Proc. Natl. Acad. Sci. USA 82 (1985) 4463–4467.

[22] N. Yamashita, E.C. Ilg, B.W. Schafer, C.W. Heizmann, T. Kosaka, Distribution of a specific calcium-binding protein of the S100 protein family, S100A6 (calcyclin), in subpopulations of neurons and glial cells of the adult rat nervous system, J. Comp. Neurol. 404 (1999) 235–257.

[23] I. Camby, N. Nagy, M.B. Lopes, B.W. Schafer, C.A. Maurage, M.M. Ruchoux, P. Murmann, R. Pochet, C.W. Heizmann, J. Brotchi, I. Salmon, R. Kiss, C. Decaestecker, Supratentorial pilocytic astrocytomas, astrocytomas, anaplastic astrocytomas and glioblastomas are characterized by a differential expression of S100 proteins, Brain Pathol. 9 (1999) 1–19.

[24] L.F. Eng, R.S. Ghirnikar, GFAP and astrogliosis, Brain Pathol. 4 (1994) 229–237.

[25] Y. Takamiya, S. Kohsaka, S. Toya, M. Otani, Y. Tsukada, Immunohistochemical studies on the proliferation of reactive astrocytes and the expression of cytoskeletal proteins following brain injury in rats, Brain Res. 466 (1988) 201–210.

[26] D. Schiffer, M.T. Giordana, P. Cavalla, M.C. Vigliani, A. Attanasio, Immunohistochemistry of glial reaction after injury in the rat: double stainings and markers of cell proliferation, Int. J. Dev. Neurosci. 11 (1993) 269–280.

[27] D. Schiffer, S. Cordera, P. Cavalla, A. Migheli, Reactive astrogliosis of the spinal cord in amyotrophic lateral sclerosis, J. Neurol. Sci. 139 (Suppl) (1996) 27–33.

[28] D. Trotti, A. Rolfs, N.C. Danbolt, R.H. Brown Jr., M.A.

Hediger, SOD1 mutants linked to amyotrophic lateral sclerosis selectively inactivate a glial glutamate transporter, Nat. Neurosci. 2 (1999) 848.
[29] E.C. Ilg, B.W. Schäfer, C.W. Heizmann, Expression pattern of S100 calcium-binding proteins in human tumours, Int. J. Cancer 68 (1996) 325–332.
[30] L. Siklós, J. Engelhardt, Y. Harati, R.G. Smith, F. Joó, S. Appel, Ultrastructural evidence for altered calcium in motor nerve terminals in amyotrophic lateral sclerosis, Ann. Neurol. 39 (1996) 203–216.
[31] J.-M. Lee, G.J. Zipfel, D.W. Choi, The changing landscape of ischaemic brain injury mechanisms, Nature 399 (1999) A7–A14.
[32] P.C. Wong, C.A. Pardo, D.R. Borchelt, M.K. Lee, N.G. Copeland, N.A. Jenkins, S.S. Sisodia, D.W. Cleveland, D.L. Price, An adverse property of a familial ALS-linked SOD1 mutation causes motor neuron disease characterized by vacuolar degeneration of mitochondria, Neuron 14 (1995) 1105–1116.
[33] L.I. Bruijn, M.W. Becher, M.K. Lee, K.L. Anderson, N.A. Jenkins, N.G. Copeland, S.S. Sisodia, J.D. Rothstein, D.R. Borchelt, D.L. Price, D.W. Cleveland, ALS-linked SOD1 mutant G85R mediates damage to astrocytes and promotes rapidly progressive disease with SOD1-containing inclusions, Neuron 18 (1997) 327–338.
[34] P.D. Kushner, D.T. Stephenson, S. Wright, Reactive astrogliosis is widespread in the subcortical white matter of amyotrophic lateral sclerosis brain, J. Neuropathol. Exp. Neurol. 50 (1991) 263–277.
[35] J.B. Levine, J. Kong, M. Nadler, Z. Xu, Astrocytes interact intimately with degenerating motor neurons in mouse amyotrophic lateral sclerosis (ALS), Glia 28 (1999) 215–224.
[36] J.D. Rothstein, M. Van Kammen, A.I. Levey, L.J. Martin, R.W. Kuncl, Selective loss of glial glutamate transporter GLT-1 in amyotrophic lateral sclerosis, Ann. Neurol. 38 (1995) 73–84.
[37] V. Parpura, P.G. Haydon, From the cover: Physiological astrocytic calcium levels stimulate glutamate release to modulate adjacent neurons, Proc. Natl. Acad. Sci. USA 97 (2000) 8629–8634.
[38] R. Donato, Functional roles of S100 proteins, calcium-binding proteins of the EF-hand type, Biochim. Biophys. Acta 1450 (1999) 191–231.
[39] J.P. Crow, J.B. Sampson, Y. Zhuang, J.A. Thompson, J.S. Beckman, Decreased zinc affinity of amyotrophic lateral sclerosis-associated superoxide dismutase mutants leads to enhanced catalysis of tyrosine nitration by peroxynitrite, J. Neurochem. 69 (1997) 1936.
[40] A.G. Estévez, J.P. Crow, J.B. Sampson, C. Reiter, Y. Zhuang, G.J. Richardson, M.M. Tarpey, L. Barbeito, J.S. Beckman, Induction of nitric oxide-dependent apoptosis in motor neurons by zinc-deficient superoxide dismutase, Science 286 (1999) 2498–2500.