Experimental Neurology 215 (2009) 77–86

Contents lists available at ScienceDirect

Experimental Neurology

journal homepage: www.elsevier.com/locate/yexnr

Voronoi-based spatial analysis reveals selective interneuron changes in the cortex of FALS mice

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ARTICLE INFO

Article history:
Received 23 June 2008
Revised 1 September 2008
Accepted 15 September 2008
Available online 26 September 2008

Keywords:
Motoneuron disease
Amyotrophic lateral sclerosis
Motor cortex
Somatosensory cortex
Parvalbumin
GABA
Spatial analysis
Cell aggregation
Cortical plasticity

ABSTRACT

The neurodegenerative disease amyotrophic lateral sclerosis affects lower motoneurons and corticospinal cells. Mice expressing human mutant superoxide dismutase (SOD)1 provide widely investigated models of the familial form of disease, but information on cortical changes in these mice is still limited. We here analyzed the spatial organization of interneurons characterized by parvalbumin immunoreactivity in the motor, somatosensory, and visual cortical areas of SOD1(G93A) mice. Cell number and sociological spatial behavior were assessed by digital charts of cell location in cortical samples, cell counts, and generation of two-dimensional Voronoi diagrams. In end-stage SOD1-mutant mice, an increase of parvalbumin-containing cortical interneurons was found in the motor and somatosensory areas (about 35% and 20%, respectively) with respect to wild-type littermates. Changes in cell spatial distribution, as documented by Voronoi-derived coefficients of variation, indicated increased tendency of parvalbumin cells to aggregate into clusters in the same areas of the SOD1-mutant cortex. Counts and coefficients of variation of parvalbumin cells in the visual cortex gave instead similar results in SOD1-mutant and wild-type mice. Analyses of motor and somatosensory areas in presymptomatic SOD1-mutant mice provided findings very similar to those obtained at end-stage, indicating early changes of interneurons in these cortical areas during the pathology. Altogether the data reveal in the SOD1-mutant mouse cortex an altered architectonic pattern of interneurons, which selectively affects areas involved in motor control. The findings, which can be interpreted as pathogenic factors or early disease-related adaptations, point to changes in the cortical regulation and modulation of the motor circuit during motoneuron disease.

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult-onset and progressive degenerative disorder which affects motoneurons of the spinal cord, brain stem and cerebral cortex. The vast majority of cases are sporadic (SALS), while approximately 10% of cases are familial (FALS). Autosomal dominant mutations in the gene encoding the enzyme superoxide dismutase (SOD)1 occur in ~20% of FALS cases. Transgenic rodents expressing the human mutant SOD1 develop a disorder resembling the phenotype and some of the histopathological features of the human disease (Kato, 2008; Turner and Talbot, 2008).

Murine FALS is characterized by an ascending course of the illness, with early involvement of lumbar motoneurons, and investigations of pathological changes have hitherto focused mostly on these cells (reviews in Bendotti and Carrì, 2004; Kato, 2008; Turner and Talbot, 2008). Spinal interneurons, which are key regulatory elements of motoneuron function, have also raised interest, and have been investigated using calcium binding proteins as markers. In SOD1 (G86R) mice, significant reduction in the number of calretinin-positive spinal interneurons, with 23.5% loss at an early symptomatic stage and 40% loss in the terminal stage, has been reported (Morrison et al., 1998). In the spinal cord of SOD1(G93A) mice, loss of interneurons containing parvalbumin (PV) precedes lower motoneuron defeat (Martin et al., 2007). These studies indicate that different neuronal populations are affected in the spinal cord of SOD1-mutant mice and point out the involvement of spinal interneuron subsets in the pathogenesis and/or progression of lower motoneuron degeneration.

Limited information is available, however, on motor-related cortical areas in murine FALS. Loss of cortical neurons retrogradely labeled from the lumbar spinal cord (Zang and Cheema, 2002) and alteration of calretinin immunoreactivity in the cortex (Chung et al., 2005) have been reported in SOD1(G93A) mice, in which decreased cortical thickness has also been described (Petrik et al., 2007), but cortical neurons, and interneurons in particular, have not been thoroughly investigated up to now. This is of relevance not only because corticospinal neurons are affected in human ALS, but also in view of changes of cortical interneurons described in other animal

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doi:10.1016/j.expneurol.2008.09.005

models of neuromuscular diseases, and in particular in *mdx* mice, which provide a model for muscular dystrophy of Duchenne type (Carretta et al., 2003, 2004; Sbriccoli et al., 1995).

On this basis, we are currently analyzing the cerebral cortex of SOD1(G93A) mice, and the present investigation focuses on PV-immunoreactive (ir) cortical interneurons. These cells represent a large subpopulation (and actually the majority) of cortical GABAergic interneurons (Celio, 1986, 1990; DeFelipe et al., 2002). PV-containing interneurons target mainly somata or proximal dendrites of pyramidal cells (Somogyi et al., 1998), display fast synaptic current kinetics (Dumitriu et al., 2007), and play a major role in mechanisms of cortical wiring and plasticity (Hensch, 2005). We here explored in murine FALS, before symptom onset and in the terminal stage of disease, the spatial organization of PV-ir cells in the motor and somatosensory areas, which notably contain corticospinal neurons. These areas were compared with the visual cortex, which does not contain corticospinal neurons in the adult brain.

Cell sociology and architectural changes in the SOD1-mutant cortex were here analyzed by the generation of 2D Voronoi diagrams, a strategy of spatial tessellation that produces polygons assigning to each neuron the free area surrounding it (Duyckaerts et al., 1994). Spatial analysis through compartment size distributions can be obtained from a wide range of random space subdivisions (Pineda et al., 2004). However, Voronoi tessellation is a well-known mathematical cellular structure which has been broadly applied to the study of cooperative behavior and topology of biological entities (Marcelpoil and Usson, 1992; Reyes and Adjouadi, 1997; Wallet and Dussert, 1997), including nerve cell sheets in experimental animals and in the human brain (Minciacchi and Granato, 1997; Moroni et al., 2007; Rivara et al., 2003). This approach has revealed complex and subtle changes in the cortex and brainstem of mutant dystrophic *mdx* mice (Carretta et al., 2001, 2004).

Preliminary results of the present study have been presented in abstract form (Minciacchi et al., 2006).

Materials and methods

Animals

A total of 22 mice were used; 11 of them carried a mutant SOD1 gene (strain designation: B6SJL-TgN(SOD1-G93A)1Gur, SOD1-mutant; Gurney et al., 1994), and 11 were wild-type (B6SJL, Wt) mice. SOD1-mutant mice, originally obtained from Jackson Laboratories (Bar Harbor, ME, USA), were identified by a polymerase chain reaction specific for human SOD1. They were sampled at 67–69 days ($n=5$) or 137–140 days of age ($n=6$); the former were presymptomatic and the latter in the terminal stage of disease, consistent with the widely documented course of the illness in this strain of mice (Turner and Talbot, 2008). Wt mice were littermates of SOD1-mutant mice of either age group. Time of disease onset (around 90 days) and its progression were assessed by careful behavioral monitoring. Before symptom onset, transgenic mice lack tremors and exhibit an intact extension reflex, as well as normal performance at the paw-grip endurance test. At end-stage, mice are unable to right themselves within 30 s of being put on their sides. The experiments were performed with the approval of the Italian Ministry of Health, following the NIH Guide for the Use and Care of Laboratory Animals, and in accordance with the European Communities Council Directive (86/609/EEC). The animals were infection-free and maintained under controlled environmental parameters with food and water *ad libitum*. Care was taken to avoid animal stress and discomfort during handling.

Tissue processing and immunohistochemistry

At the time of sacrifice, the animals were deeply anesthetized with Nembutal (50 mg/kg i.p.) and perfused transcardially with phosphate-buffered saline (0.01 M, pH 7.4; PBS) followed by 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4. The brains were dissected out, soaked overnight in 30% buffered sucrose at 4 °C, and cut on a freezing microtome into 30 μm-thick coronal sections. All sections through the cerebral cortex were collected in two series, destined to the immunohistochemical study of PV and to thionin staining (0.25% thionin solution; Luna, 1968), respectively.

Sections from SOD1-mutant mice and age-matched Wt ones were processed for PV immunohistochemistry together and in the same solutions. They were incubated in 3% normal goat serum for 1 h and then in monoclonal mouse anti-PV antibodies (Sigma P3088; Sigma-Aldrich Co., St. Louis, MO, USA), diluted 1:10,000 in 0.3% Triton X-100/3% normal goat serum in PBS, for 36 h at 4 °C. The sections were then incubated in goat anti-mouse biotinylated secondary antibodies (dilution 1:200; Sigma-Aldrich) in 0.3% Triton X-100 in PBS for 1 h, followed by the avidin–biotin procedure (ABC kit; Pierce, Rockford, IL, USA), and finally reacted with 3-3′ diaminobenzidine dihydrochloride (Sigma-Aldrich). Reacted sections were mounted on gelatin-coated slides, dehydrated, and coverslipped (Eukitt, Kindler, Germany). Thionin-stained sections were used to determine cortical cytoarchitecture. Some sections from the series collected for thionin staining were used as control for the immunohistochemical procedure. These sections were processed with omission of primary antibody or incubation in normal mouse IgGs (with corresponding protein concentrations and incubation time) diluted in 0.3% Triton X-100/3% in normal goat serum in PBS. No immunostaining was seen in this material.

Nomenclature and boundaries for the motor, somatosensory, and visual cortical areas are according to Verne and Caviness (1976) and Paxinos and Franklin (2001). For layering of the motor cortex, we adopted the subdivision identifying layers V-A and V-B as homologous to layers IV and V, respectively, of the somatosensory and visual cortical areas (Brecht et al., 2004; Caviness, 1975; Weiler et al., 2008).

Table 1
Number of parvalbumin-positive cells (*n*, mean per sample ± standard deviation) and Voronoi-derived coefficients of variation (CVs) in the analyzed cortical areas of SOD1-mutant and wild-type (Wt) mice

		Motor cortex		Somatosensory cortex		Visual cortex	
		n cells	CVs	*n* cells	CVs	*n* cells	CVs
Symptomatic	Wt	56.40±2.50	0.44±0.01	97.81±2.70	0.56±0.04	72.08±2.12	0.47±0.02
	SOD1-mutant	76.76±2.11	0.53±0.04	117.74±2.77	0.59±0.03	71.39±1.84	0.48±0.02
	Wt *vs* SOD1-mutant*	231.61***	27.59***	159.82***	3.48	0.37	1.00
Presymptomatic	Wt	57.22±1.80	0.44±0.02	95.68±2.58	0.55±0.02		
	SOD1-mutant	78.33±2.32	0.52±0.02	119.92±2.47	0.61±0.02		
	Wt *vs* SOD1-mutant**	267.56***	62.33***	290.76***	25.77****		

* ANOVA results are given as $F1,10$ values.
** ANOVA results are given as $F1,8$ values.
*** Bonferroni *post-hoc* test following ANOVA: $P<0.001$.
**** Bonferroni *post-hoc* test following ANOVA: $P=0.001$.