Neuroscience Letters 666 (2018) 1–4

Contents lists available at ScienceDirect

Neuroscience Letters

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

Research article

A potential role for neuronal connexin 36 in the pathogenesis of amyotrophic lateral sclerosis

Andrei B. Belousov[a,*], Hiroshi Nishimune[b], Janna V. Denisova[a], Joseph D. Fontes[c]

[a] Departments of Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, KS, USA
[b] Departments of Anatomy and Cell Biology, University of Kansas Medical Center, Kansas City, KS, USA
[c] Departments of Biochemistry and Molecular Biology, University of Kansas Medical Center, Kansas City, KS, USA

ARTICLE INFO

Keywords:
Gap junctions
Connexin 36
Amyotrophic lateral sclerosis
Spinal cord
Neurodegenerative diseases

ABSTRACT

Neuronal gap junctional protein connexin 36 (Cx36) contributes to neuronal death following a range of acute brain insults such as ischemia, traumatic brain injury and epilepsy. Whether Cx36 contributes to neuronal death and pathological outcomes in chronic neurodegenerative diseases, such as amyotrophic lateral sclerosis (ALS), is not known. We show here that the expression of Cx36 is significantly decreased in lumbar segments of the spinal cord of both human ALS subjects and SOD1^{G93A} mice as compared to healthy human and wild-type mouse controls, respectively. In purified neuronal cultures prepared from the spinal cord of wild-type mice, knockdown of Cx36 reduces neuronal death caused by overexpression of the mutant human SOD1-G93A protein. Taken together, these data suggest a possible contribution of Cx36 to ALS pathogenesis. A perspective for the use of blockers of Cx36 gap junction channels for ALS therapy is discussed.

1. Introduction

In the mammalian central nervous system, direct intercellular communication between neighboring neurons occurs through electrical synapses (gap junctions; GJ). Using animal models of acute neuronal injury such as brain ischemia, traumatic brain injury, epilepsy and glutamate-mediated excitotoxicity, we have shown previously that genetic and/or pharmacological blockade of neuronal, connexin 36 (Cx36)-containing GJs is unambiguously neuroprotective [1–4]. In the ischemic mouse cortex *in vivo* and *in vitro*, we also demonstrated a transient increase (at 2–3 h post-ischemia) with the following profound decrease (at 24 h post-ischemia) in the expression of Cx36 [3]. The delayed Cx36 downregulation overlapped with massive post-ischemic death of neurons. Altogether, our data suggested a critical role for Cx36 in neuronal death following acute brain injury. A modified model of glutamate-mediated excitotoxicity, where neuronal GJs are intimately involved, has been proposed [5].

Amyotrophic lateral sclerosis (ALS), is a progressive neurodegenerative disease characterized by preferential degeneration of upper and lower motor neurons, gradual decline of muscle strength and death within 3–5 years after the first symptoms [6]. Mutations of the superoxide dismutase 1 (SOD1) gene are frequently associated with the familial form of ALS [7]. Multiple mechanisms for the contribution of SOD1 variants in ALS-related motor neuron degeneration have been suggested [8,9]. However, whether as in case of the acute brain injury, Cx36 plays a role in neuronal death during ALS and other chronic neurodegenerative diseases, is not known. In the present study, using purified neuronal cultures prepared from the wild-type (WT) mouse spinal cord, we determined whether genetic knockdown of Cx36 reduces neuronal death caused by overexpression of the human SOD1-G93A mutant protein. Using spinal cord samples from ALS human subjects and SOD1^{G93A} mice (a commonly used animal model of familial ALS), we also tested the hypothesis that the expression of Cx36 is decreased at the late stages of ALS, *i.e.*, at the time when neuronal degeneration is observed [6,10–13].

2. Materials and methods

Postmortem human samples of the lumbar spinal cord were obtained from the NIH NeuroBioBank (see Acknowledgements). SOD1^{G93A} mice (on the C57BL/6 background) were originally obtained from the Jackson Laboratory (stock # 004435). The use of animal subjects in these experiments was approved by the University of Kansas Medical Center Animal Care and Use Committee. The experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory. All studies were conducted blindly.

Western blot experiments were performed using the approaches and antibodies as described in detail [14]. In addition, rabbit anti-Cx45

* Corresponding author at: Department of Molecular and Integrative Physiology, University of Kansas Medical Center, 2146 W. 39th Avenue, Kansas City, KS 66160, USA.
E-mail address: abelousov@kumc.edu (A.B. Belousov).

https://doi.org/10.1016/j.neulet.2017.12.027
Received 13 October 2017; Received in revised form 4 December 2017; Accepted 11 December 2017
Available online 12 December 2017
0304-3940/

primary antibody was obtained from Invitrogen (Carlsbad, CA, USA; cat.# 407000). Band optical density was determined using a direct detection with Quantity One software (Bio-Rad, USA). Optical density signals were normalized relative to tubulin and normalized values were compared to controls (set at 1.0). Tubulin levels per unit of total protein did not vary significantly among samples. The following postmortem samples of the lumbar spinal cord were utilized from healthy human subjects and those diagnosed with ALS (gender/years of age): controls – M/60, M/74, F/54; ALS – M/60, M/78, F/54. In addition, three WT mice (C57BL/6) and three SOD1^{G93A} mice were used (gender/days of age): WT – M/172, M/148, M/148; SOD1^{G93A} – M/139, M/150, M/150. The ALS mice were sacrificed when they were unable to right themselves within 30 s when placed on their sides and, thus, were at the end stage of disease. The mice were genotyped using qPCR based protocol to confirm the transgene copy number.

To knockdown Cx36, we used a lentivirus expressing Cx36-targeted small hairpin RNA (shRNA-Cx36) that we designed previously (shRNA1-Cx36 from [4]). A lentivirus expressing shRNA directed against *Cypridina* luciferase (shRNA-LUC; [4]) was used as a control. A multiplicity of transduction of 20 for both lentiviral vectors was employed. Suppression of Cx36 protein by shRNA-Cx36 was confirmed using western blots (see Results).

An expression plasmid for (Myc-DDK-tagged)-human soluble SOD1 was purchased from Origene, Inc. (Rockville, MD, USA; cat.# RC200725; Accession number NM_000454.4). The codon for glycine at position 93 was mutated to a codon for alanine using site-directed mutagenesis (QuikChange II kit; Agilient, Inc., Santa Clara, CA, USA). The SOD1 cDNA in both the purchased plasmid and the G93A mutation were fully sequenced to confirm identity. An empty vector (VECT) was used as a transfection control. WT mouse spinal cord cultures were transfected with Lipofectamine 2000 (Life Technologies, Inc., Carlsbad, CA) and plasmid DNA (2 μg plasmid, 2 μl Lipofectamine 2000 combined in a total volume of 200 μl media, per well in 24-well plates containing primary mouse neurons cultured as outlined below). To confirm the expression of the SOD1 and SOD1-G93A mutant we employed western blots and an anti-FLAG antibody (see Results).

Purified neuronal cultures containing ~95% neurons were prepared (as we described [14]) from the spinal cord of embryonic day 18–19 WT mice. On day *in vitro* 3 (DIV3), the cultures were singly transduced with shRNA-LUC or shRNA-Cx36 (as we described [4]). On DIV4, the cultures also were singly transfected with the control plasmid or plasmids inducing human SOD1 or SOD1-G93A mutant. On DIV10, methyl thiazolyl tetrazolium (MTT) assay was conducted. The MTT tests were designed to specifically analyze the death of neurons as we described previously [3].

Data were analyzed using the two-tailed unpaired Student's *t*-test or ANOVA with *post hoc* Tukey and InStat software (GraphPad Software, San Diego, CA, USA). Data are reported as mean ± SE for the number of samples indicated.

3. Results

We tested whether the expression of connexins, and particularly Cx36, was altered during the late stages of ALS. Western blot experiments were conducted in postmortem samples of the lumbar spinal cord obtained from the healthy human subjects and diagnosed ALS patients (see Materials and methods). We observed a significant downregulation of the expression of neuronal Cx36 protein in the ALS spinal cord (Fig. 1A). In contrast, the expression of non-neuronal Cx43, that is found in astrocytes [15] and activated microglia [16], was increased during ALS (Fig. 1B). Cx45, that also may be expressed by neurons [17], was detected in neither control nor ALS conditions (Fig. 1C). Essentially identical results were obtained in the spinal cord samples utilized from WT and SOD1^{G93A} mice (Fig. 1D–F). Together, these findings indicate that neuronal Cx36 expression is decreased in the spinal cord at the late stages of ALS, while astroglial Cx43 is upregulated.

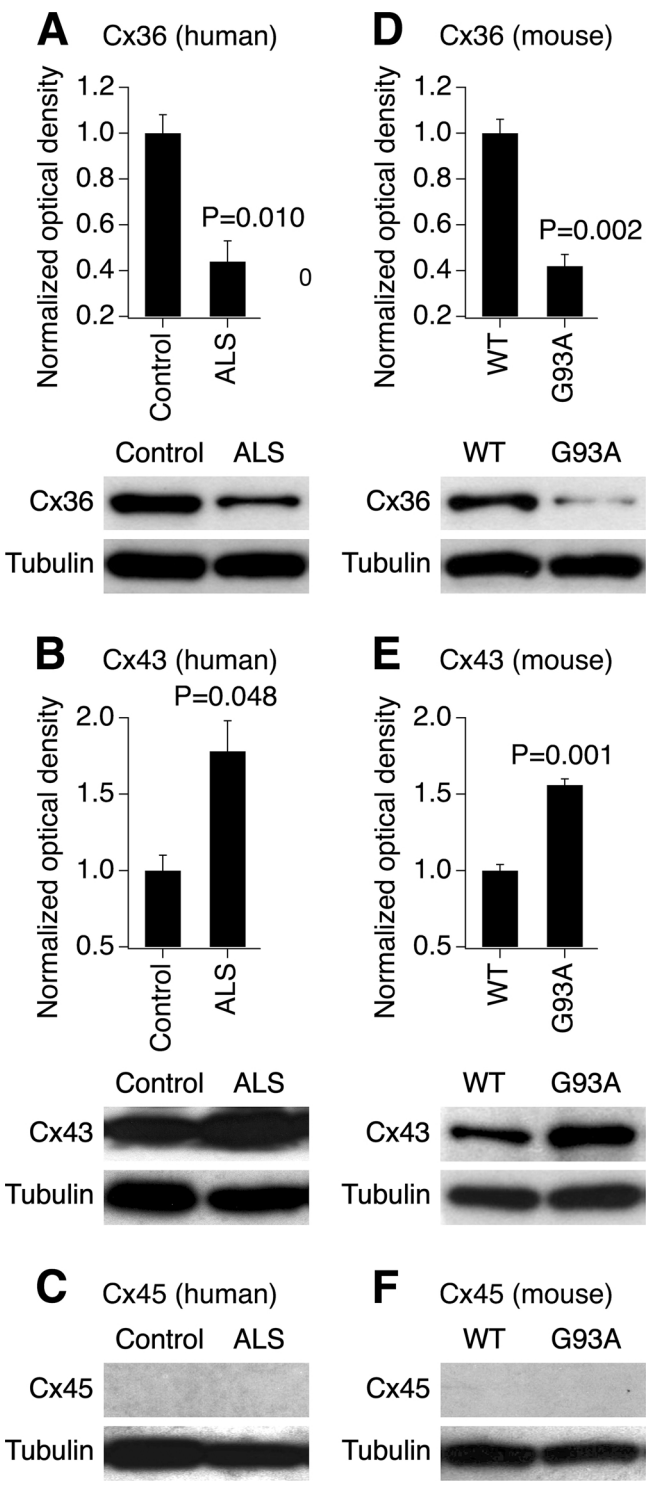

Fig. 1. Expression of neuronal Cx36 decreases and glial Cx43 increases in ALS conditions. Representative blots and statistical data from western blot experiments in the lumbar spinal cord samples obtained from human subjects (A–C) and mice (D–F) are shown. The analysis was done for the expression of Cx36 (A, D), Cx43 (B, E) and Cx45 (C, F). The expression of Cx45 was not detected. In all graphs, optical density signals are normalized relative to tubulin and compared to the control (healthy human subjects or WT mice). Statistical analysis: two-tailed unpaired Student's *t*-test relative to the control; $n = 3$ per group in all groups; data are shown as mean ± SE. G93A, SOD1^{G93A} mice.

Previous studies have shown that transfection of cultured rat spinal cord motor neurons with a plasmid inducing SOD1-G93A mutant protein triggers mitochondrial fragmentation and neuronal death [9]. However, this does not occur in cultures transfected with a plasmid