Neurobiology of Disease 64 (2014) 48–59

Contents lists available at ScienceDirect

Neurobiology of Disease

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

Knocking down metabotropic glutamate receptor 1 improves survival and disease progression in the *SOD1^{G93A}* mouse model of amyotrophic lateral sclerosis ☆

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ARTICLE INFO

Article history:
Received 31 July 2013
Revised 17 October 2013
Accepted 12 November 2013
Available online 19 December 2013

Keywords:
Amyotrophic lateral sclerosis
SOD1^{G93A} mouse
Metabotropic glutamate type 1 receptor
Metabotropic glutamate type 5 receptor
Metabotropic glutamate type 1 receptor knocking down
Glutamate transmission
Disease development

ABSTRACT

Amyotrophic lateral sclerosis (ALS) is a late-onset fatal neurodegenerative disease reflecting degeneration of upper and lower motoneurons (MNs). The cause of ALS and the mechanisms of neuronal death are still largely obscure, thus impairing the establishment of efficacious therapies. Glutamate (Glu)-mediated excitotoxicity plays a major role in MN degeneration in ALS. We recently demonstrated that the activation of Group I metabotropic Glu autoreceptors, belonging to both type 1 and type 5 receptors (mGluR1 and mGluR5), at glutamatergic spinal cord nerve terminals, produces excessive Glu release in mice over-expressing human superoxide-dismutase carrying the G93A point mutation (*SOD1^{G93A}*), a widely used animal model of human ALS. To establish whether these receptors are implicated in ALS, we generated mice expressing half dosage of mGluR1 in the *SOD1^{G93A}* background (*SOD1^{G93A}Grm1$^{crv4/+}$*), by crossing the *SOD1^{G93A}* mutant mouse with the *Grm1$^{crv4/+}$* mouse, lacking mGluR1 because of a spontaneous recessive mutation. *SOD1^{G93A}Grm1$^{crv4/+}$* mice showed prolonged survival probability, delayed pathology onset, slower disease progression and improved motor performances compared to *SOD1^{G93A}* mice. These effects were associated to reduction of mGluR5 expression, enhanced number of MNs, decreased astrocyte and microglia activation, normalization of metallothionein and catalase mRNA expression, reduced mitochondrial damage, and decrease of abnormal Glu release in spinal cord of *SOD1^{G93A}Grm1$^{crv4/+}$* compared to *SOD1^{G93A}* mice. These results demonstrate that a lower constitutive level of mGluR1 has a significant positive impact on mice with experimental ALS, thus providing the rationale for future pharmacological approaches to ALS by selectively blocking Group I metabotropic Glu receptors.

Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neuromuscular disorder characterized by degeneration of cortical, brainstem and spinal motoneurons (MNs) leading to muscle wasting, weakness and spasticity. ALS has an incidence of approximately 1–2 new cases per 100,000 individuals every year and is most commonly sporadic, although familial forms have been reported in about 10% of cases (Andersen and Al-Chalabi, 2011). The first identified ALS-linked gene is the superoxide dismutase-1 (SOD1) that accounts for about 20% of patients with familial ALS (Birve et al., 2010; Rosen et al., 1993). So far, at least other fifteen genes involved in different cellular pathways have been associated to ALS, thus indicating that, even though pathogenic mechanisms are still elusive, multiple cellular events contribute to the disease (Andersen and Al-Chalabi, 2011). These events include oxidative stress,

Abbreviations: ALS, amyotrophic lateral sclerosis; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole propionate; [^{3}H]D-Asp, [^{3}H]D-aspartate; CAT, catalase; 3,5-DHPG, (S)-3,5-dihydroxyphenylglycine; Gapdh, Glyceraldehyde 3-phosphate dehydrogenase; Glu, glutamate; LTR, long terminal repeat; mGluR1, metabotropic glutamate receptor 1; mGluR5, metabotropic glutamate receptor 5; MN, motoneuron; MT, metallothionein; NMDA, N-methyl-D-aspartate; PB, phosphate buffer; PFA, paraformaldehyde.

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Available online on ScienceDirect (www.sciencedirect.com).

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http://dx.doi.org/10.1016/j.nbd.2013.11.006

mitochondrial dysfunction, protein aggregation, impaired anterograde and retrograde transport, neuroinflammation, dysregulated RNA signaling, and glutamate(Glu)-mediated excitotoxicity (Cleveland et al., 1996; Ferraiuolo et al., 2011).

Glu exerts its actions through activation of ionotropic and metabotropic receptors. Three ionotropic receptor families, namely the Ca^{2+}-permeant N-methyl-D-aspartate (NMDA) receptor, and the preferentially Na^{+}-permeant α-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) and kainate receptors, exist as multiple heteromers formed by the co-assembly of different subunits (Conti and Weinberg, 1999; Dingledine et al., 1999). Metabotropic glutamate receptors (mGluR) are also heterogeneous and classified into three groups, based on their sequence homology, signaling and pharmacology (Conn and Pin, 1997; Nicoletti et al., 2011). Group I mGluRs, comprising mGluR1 and mGluR5, are excitatory because of positive coupling to phosphatidylinositol breakdown (Conn and Pin, 1997; De Blasi et al., 2001; Ferraguti et al., 2008). Thus, hyper-activation of Glu receptors may lead to an excessive increase of intracellular calcium due to either its entry through ionotropic Glu receptors and/or to its release from intracellular stores, mediated by Group I mGluRs and contributing to excitotoxicity and cell death (Doble, 1999).

Evidence implicating Glu-mediated excitotoxicity in ALS is mainly based on the presence of elevated levels of extracellular Glu in a high percentage of sporadic and familial ALS patients (Perry et al., 1990), on the reduced expression of the Glu transporter type 1 (GLT1) in the affected areas of the CNS (Rothstein et al., 1992, 1995) and on the observation that ameliorating excitotoxicity is to date the only clinically-adopted strategy to slow down disease progression in ALS (Cheah et al., 2010).

The molecular mechanisms of MN degeneration in ALS have been largely investigated in the subtype of disease caused by SOD1 mutations. Mouse models expressing mutated SOD1 reproduce most of the pathogenic processes underlying human ALS, including unfolded protein response activation (Wang et al., 2011a), altered AMPA receptor subunit expression (Tortarolo et al., 2006), reduced expression and activity of GLT1 (Boston-Howes et al., 2006), loss of astrocytic regulation GluR2 expression in MNs (Van Damme et al., 2007), impaired calcium buffering in mitochondria (Damiano et al., 2006) defective axonal transport (Bilsland et al., 2010), and Glu mediated excitotoxicity (Heath and Shaw, 2002). In addition, our recent studies with mice expressing human SOD1 carrying the G93A point mutation ($SOD1^{G93A}$), the most widely used animal model for human ALS, indicate that Glu release is abnormally high in the spinal cord of these animals upon exposure to different releasing stimuli, including nerve terminal depolarization (Milanese et al., 2011; Raiteri et al., 2004). We have recently shown that activation of presynaptic autoreceptors, belonging to mGluR1 and mGluR5 types, by submicromolar concentrations of the mGluR1/5 agonist 3,5-DHPG, promoted an excessive Glu release in the spinal cord of $SOD1^{G93A}$ mice compared to controls (Giribaldi et al., 2013).

Here, we sought to explore whether excessive Group I mGluR activity plays a role in the pathogenesis of ALS. To this end we crossed $SOD1^{G93A}$ mice with mice lacking the mGluR1 ($Grm1^{crv4/+}$; Conti et al., 2006) obtaining double mutants expressing the $SOD1^{G93A}$ mutated gene and half dosage of mGluR1 ($SOD1^{G93A}Grm1^{crv4/+}$).

Materials and methods

Animals

$Grm1^{crv4}$ mice line carrying a recessive loss-of-function mutation (*crv4*) in the gene (*Grm1*) coding for mGluR1 were used to prepare $SOD1^{G93A}Grm1^{crv4/+}$ double mutants. The *crv4* mutation is a spontaneous recessive mutation occurring in the BALB/c/Pas inbred strain. It consists of an insertion of a retrotransposon LTR (Long Terminal Repeat) fragment occurring in intron 4 of the *Grm1* gene and causing the disruption of the gene splicing and the absence of the receptor protein (Conti et al., 2006). Affected ($Grm1^{crv4/crv4}$) and control ($Grm1^{+/+}$) mice were maintained on the same genetic background by intercrossing $Grm1^{crv4/+}$ mice. The genotype of $Grm1^{crv4}$mice was identified by PCR using specific primers as already reported (Conti et al., 2006). B6SJL-Tg(SOD1*G93A)1Gur mice expressing high copy number of mutant human SOD1 with a Gly93Ala substitution ($SOD1^{G93A}$ mice) (Gurney et al., 1994) were originally obtained from Jackson Laboratories (Bar Harbor, ME, USA). Transgenic male mice were crossed with background-matched B6SJL wild-type females and selective breeding maintains the transgene in the hemizygous state. Transgenic mice are identified analyzing tissue extracts from tail tips as previously described (Stifanese et al., 2010). $SOD1^{G93A}$ male mice (on a mixed C57BL6-SJL background) were bred with $Grm1^{crv4/+}$ females (BALB/c background) to generate double-mutants carrying the $Grm1^{crv4/+}$ heterozygous mutation and the SOD1*G93A transgene ($SOD1^{G93A}Grm1^{crv4/+}$). All experiments were conducted on littermates derived from this last breeding (Fig. 1A). Animals were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) with a regular 12 h–12 h light–dark cycle (light 7 AM–7 PM), throughout the experiments. Food (type 4RF21 standard diet obtained from Mucedola, Settimo Milanese, Milan, Italy) and water were freely available. All experiments were carried out in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable results. Sexes were balanced in each experimental group to avoid bias due to sex-related intrinsic differences in disease severity. For experimental use animals were killed at symptomatic stage of disease (19 weeks). A total number of 30 WT, 55 $SOD1^{G93A}$, 55 $SOD1^{G93A}Grm1^{crv4/+}$, and 30 $Grm1^{crv4/+}$ mice were used in this study.

Survival and motor performance

Survival time was identified as the time at which mice were unable to right itself within 20 seconds when placed on their side. The effects of the genetic manipulation on the progression of the disease symptoms were analyzed by Rotarod task and motor deficits. Each clinical test registration was started on day 90 and data were recorded three times a week, until death, in WT, $SOD1^{G93A}$, $SOD1^{G93A}Grm1^{crv4/+}$, and $Grm1^{crv4/+}$ mice. Tests were performed in randomized order by blinded observers. *Rotarod test*: starting on day 90, the time for which an animal could remain on the rotating cylinder was measured using an accelerating Rotarod apparatus (Rota-Rod 7650; Ugo Basile, Comerio, Italy). In this procedure the rod rotation gradually increases in speed from 4 to 40 rpm over the course of 5 min. The time that the mice stayed on the rod until falling off was recorded. Before registration animals were trained for 10 days. *Motor deficits*: mice were rated for disease progression by scoring the extension reflex of hind limbs and the gait. In the extension reflex test, animals were evaluated by observing the hind limb posture when suspended by the tail. Gait deficits were measured by observing mice in an open field. Motor deficits were rated using a 5 point score scale (5, no sign of motor dysfunction; 0, complete impairment) as previously described (Uccelli et al., 2012). *Body weight*: Body weight was measured immediately before behavioral tests. Disease onset was defined retrospectively as the time when mice reached peak of body weight (Boillée et al., 2006b). Animals used in the clinical tests were not used for other experiments.

Histological studies

Tissue preparation

Nineteen-weekold WT, $SOD1^{G93A}$, $SOD1^{G93A}Grm1^{crv4/+}$, and$Grm1^{crv4/+}$ mice were anesthetized with choral hydrate (300 mg/kg) and perfused