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Altered mechanisms underlying the abnormal glutamate release in amyotrophic lateral sclerosis at a pre-symptomatic stage of the disease

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ABSTRACT

Abnormal Glu release occurs in the spinal cord of SOD1^{G93A} mice, a transgenic animal model for human ALS. Here we studied the mechanisms underlying Glu release in spinal cord nerve terminals of SOD1^{G93A} mice at a pre-symptomatic disease stage (30 days) and found that the basal release of Glu was more elevated in SOD1^{G93A} with respect to SOD1 mice, and that the surplus of release relies on synaptic vesicle exocytosis. Exposure to high KCl or ionomycin provoked Ca^{2+}-dependent Glu release that was likewise augmented in SOD1^{G93A} mice. Equally, the Ca^{2+}-independent hypertonic sucrose-induced Glu release was abnormally elevated in SOD1^{G93A} mice. Also in this case, the surplus of Glu release was exocytotic in nature. We could determine elevated cytosolic Ca^{2+} levels, increased phosphorylation of Synapsin-I, which was causally related to the abnormal Glu release measured in spinal cord synaptosomes of pre-symptomatic SOD1^{G93A} mice, and increased phosphorylation of glycogen synthase kinase-3 at the inhibitory sites, an event that favours SNARE protein assembly. Western blot experiments revealed an increased number of SNARE protein complexes at the nerve terminal membrane, with no changes of the three SNARE proteins and increased expression of synaptotagmin-1 and β-Actin, but not of an array of other release-related presynaptic proteins. These results indicate that the abnormal exocytotic Glu release in spinal cord of pre-symptomatic SOD1^{G93A} mice is mainly based on the increased size of the readily releasable pool of vesicles and release facilitation, supported by plastic changes of specific presynaptic mechanisms.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neuromuscular disorder characterized by degeneration of cortical, brainstem and spinal motor neurons (MNs) leading to muscle wasting, weakness and spasticity (Brown, 1995; Eisen, 2009). Although the clinical outcome in ALS is defined by MN degeneration, the disease is non-cell autonomous, also involving astrocytes, microglia and oligodendrocytes (Boillée et al., 2006; Haidet-Phillips et al., 2011; Ilieva et al., 2009).

ALS has an incidence of 2–3 new cases/year per 100,000 individuals and it is most commonly sporadic, although familial forms represent about 10% of patients (Andersen and Al-Chalabi, 2011). The first identified ALS-linked gene was superoxide dismutase-1 (SOD1) that accounts for about 20% of patients with familial ALS (Birve et al., 2010; Mulder et al., 1986; Rosen, 1993). So far, at least fifteen more genes involved in different cellular pathways have been associated to ALS (Andersen and Al-Chalabi, 2011), thus indicating that multiple cellular events contribute to the disease. They include oxidative stress, mitochondrial dysfunction, protein aggregation, impaired axonal transport, neuroinflammation,

Abbreviations: α/β-SNAP, α/β soluble NSF attachment proteins; ALS, amyotrophic lateral sclerosis; BoNt-C1, Botulinum Neurotoxin C1; β-Tub III, β-Tubulin III; $[Ca^{2+}]_c$, cytosolic calcium concentration; CaMK-II, Ca^{2+}/calmodulin-dependent protein kinase II; DL-TBOA, DL-threo-beta-benzyloxyaspartate; GSK-3, glycogen synthase kinase 3; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; [^{3}H]D-Asp, [^{3}H]D-Aspartate; mAbs, monoclonal antibodies; MNs, motor neurons; Munc-13, mammalian uncoordinated-13; Munc-18, mammalian uncoordinated-18; NSF, N-ethylamide sensitive fusion protein; Rab-3A, Ras-related protein 3A; RRP, readily releasable pool; SNAP-25, synaptosome-associated protein of 25 kDa; SOD1, superoxide dismutase-1; SNARE, soluble NSF attachment protein receptors; Stx-1, syntaxin 1; Syn-I, Synapsin-I; Syph-1, synaptophysin 1; Syt-1, synaptotagmin 1; VAMP-2, vesicular associate membrane protein 2.

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dysregulated RNA signaling, immunological imbalance and glutamate(Glu)-mediated excitotoxicity (Cleveland et al., 1996; King et al., 2016; Morrison and Morrison, 1999; Peters et al., 2015; Tan et al., 2014; Van Den Bosch et al., 2006).

Strong evidence supports the notion that altered excitatory transmission in spinal cord and excitotoxicity, based on high levels of extra-cellular Glu and abnormal function of postsynaptic Glu receptors at lower MNs, play a major role in disease progression and cell death (Kuner et al., 2005; Shaw and Eggett, 2000; Tortarolo et al., 2006; Van Damme et al., 2005; Van Den Bosch et al., 2000; Wuolikainen et al., 2011). Elevation of Glu concentration in plasma and cerebrospinal fluid has been documented in ALS patients (Perry et al., 1990; Rothstein et al., 1990; Shaw et al., 1995; Spreux-Varoquaux et al., 2002; Wuolikainen et al., 2011) and impaired clearance of Glu by astrocyte uptake, due to reduced expression of Glu transporter 1, has been proposed as a cause of neurotoxicity (Cleveland and Rothstein, 2001; Rothstein et al., 1995), although the hypothesis that Glu transport dysfunction is a primary event for MN death in spinal cord has been disputed (Corona and Tapia, 2004; Tovar-Y-Romo et al., 2009).

More recent studies of our group with mice expressing human SOD1 carrying the G93A point mutation ($SOD1^{G93A}$) indicated that neuronal Glu release, under basal condition and upon exposure to different releasing stimuli, including nerve terminal depolarization, is abnormally high in the spinal cord of these animals at the late stage of disease. We found that increased cytosolic calcium concentration ($[Ca^{2+}]_c$), the associated over-activation of Ca^{2+}/calmodulin-dependent protein kinase II (CaMK-II), which has been already shown in sporadic ALS patients (Hu et al., 2003) and, in turn, the phosphorylation of Synapsin I (Syn-I), an event that contributes to fill up the readily releasable pool (RRP) of vesicles and to boost vesicles fusion, support the stimulus-evoked Glu release (Milanese et al., 2011). The higher exocytotic release of Glu is detectable also at the early-symptomatic and pre-symptomatic stages of the disease. This precociousness fosters the hypothesis that the increased release of Glu represents a pivotal factor in the pathology rather than a consequence of disease progression, pointing to this mechanism in the panorama of the ALS causes.

Whereas the mechanisms supporting excessive Glu release at the late stage of the disease have been, at least in part, elucidated no studies are available at pre-symptomatic stages. In this work, we report that the abnormal Glu release in 30 day-old pre-symptomatic $SOD1^{G93A}$ mice is exocytotic and that augmentation of $[Ca^{2+}]_c$ and of the number of soluble NSF attachment protein receptors (SNARE) complexes seem to be major causes for the abnormal neurotransmitter release. Moreover, we found selective molecular changes in spinal cord nerve terminals, including increased phosphorylation/activation of Syn-I, increased expression of synaptotagmin-1 (Syt-1) and β-Actin, inhibition of glycogen synthase kinase 3 (GSK-3), all of which may be related to the abnormal Glu release.

2. Materials and methods

2.1. Animals

B6SJL-TgN SOD1/G93A(+)1Gur mice expressing high copy number of mutant human SOD1 with a $Gly^{93}Ala$ substitution [$SOD1^{G93A}$] and B6SJL-TgN (SOD1)2Gur mice expressing wild-type human SOD1 (SOD1) (Gurney et al., 1994) were originally obtained from Jackson Laboratories (Bar Harbor, ME) and bred at the animal facility of the Pharmacology and Toxicology Unit, Department of Pharmacy, University of Genoa. Transgenic animals have been crossed with background-matched B6SJL wild type female and selective breeding maintained each transgene in the hemizygous state. All transgenic mice were identified analysing extracts from tail tips (homogenized in phosphate-buffer saline, freeze/thawed twice and centrifuged at 23,000 ×*g* for 15 min at 4 °C) by staining for SOD1 after polyacrylamide gel electrophoresis (10% resolving and 4% stacking) (Laemmli, 1970; Stifanese et al., 2010). Animals were housed at constant temperature (22 ± 1 °C) and relative humidity (50%) under a regular dark-light schedule (light on 7 a.m. to 7 p.m.). Food and water were freely available. Animals of either sex were randomly divided into different experimental groups. All experiments were carried out in accordance with the guidelines established by the European Communities Council (EU Directive 114 2010/63/EU for animal experiments published on September 22nd, 2010) and with the Italian D.L. n. 26/2014, and were approved by the Italian Ministry of Health (prot. no. 31754-3). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable results. All the performed experiments using animals comply with the ARRIVE guidelines.

2.2. Preparation of synaptosomes

Animals were euthanized and the whole spinal cord rapidly removed. Synaptosomes were prepared essentially as described previously (Raiteri et al., 2008). The tissue was homogenized in 14 volumes of 0.32 M sucrose, buffered at pH 7.4 with Tris-HCl, using a glass-Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged (5 min, 1000 ×*g* at 4 °C) to remove nuclei and debris and the supernatant was gently stratified on a discontinuous Percoll® (Sigma-Aldrich, St Louis, Missouri, USA) gradient (2, 6, 10 and 20% v/v in Tris-buffered 0.32 M sucrose). After centrifugation at 33,500 ×*g* for 5 min, the layer between 10 and 20% Percoll® (synaptosomal fraction) was collected and washed by centrifugation at 20,000 × *g* for 15 min in pysiological medium. The synaptosomal precipitate was then resuspended in physiological medium having the following compositions (mM): NaCl, 140; KCl, 3; $MgSO_4$, 1.2; NaH_2PO_4, 1.2; $NaHCO_3$, 5; $CaCl_2$, 1.2; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 10; glucose, 10; pH 7.4 for $[Ca^{2+}]_c$ determination and release experiments or in lysis buffer for western blotting. Protein was measured according to Bradford (1976) using bovine serum albumin (Sigma-Aldrich, St Louis, Missouri, USA) as a standard. All the reagents were of laboratory grade.

2.3. Ca^{2+} determination

$[Ca^{2+}]_c$ was determined in spinal cord synaptosomes using the fluorescent dye fura-2/AM (Grynkiewicz et al., 1985). Synaptosomes were incubated for 40 min at 37 °C, while gently shaking, in the HEPES-containing physiological medium, in the presence of 20 μM of $CaCl_2$ and 5 μM fura-2/AM and 0.5% dimethyl sulfoxide (DMSO; Sigma-Aldrich, St Louis, Missouri, USA). Synaptosomes, incubated in the presence of 0.5% DMSO only, were used to measure auto-fluorescence. After extra-synaptosomal fura-2/AM removal, the pellets were resuspended in ice-cold standard or Ca^{2+}-free HEPES-buffered medium, divided into 200 μl aliquots (200 μg protein/sample), and stored on ice until use. Measures were obtained within 2 h. Synaptosomes were diluted in HEPES-buffered medium containing the appropriate Ca^{2+} concentration (final volume 2 ml) and equilibrated at 37 °C for 15 min. The measurements were made at 37 °C under continuous stirring using an RF-5301PC dual wavelength spectrofluorophotometer (Shimadzu Corporation, Milan, Italy) by alternating the excitation wavelengths of 340 and 380 nm. Fluorescent emission was monitored at 510 nm. Basal fluorescence was recorded for 1 min, then synaptosomes were exposed to KCl for additional 2 min. Calibration of the fluorescent signal was performed at the end of each measure by adding 10 μM ionomycin in the presence of $CaCl_2$ to obtain F_{max}, followed by 10 mM ethylene glycol tetraacetic acid (EGTA); adjusted to pH 8.0 with 3 mM; Tris) to obtain F_{min}. After correcting for extracellular dye, $[Ca^{2+}]_c$ was calculated by the equation of Grynkiewicz et al. (1985), using a K_D of 224 nM for the Ca^{2+}-fura-2 complex. All the reagents were of laboratory grade.