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Intrathecal infusion of a Ca^{2+}-permeable AMPA channel blocker slows loss of both motor neurons and of the astrocyte glutamate transporter, GLT-1 in a mutant SOD1 rat model of ALS

Hong Z. Yin [a], Darryl T. Tang [a], John H. Weiss [a,b,*]

[a] *Department of Neurology, University of California, Irvine, CA, USA*
[b] *Department of Anatomy and Neurobiology, University of California, Irvine, CA, USA*

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Abstract

Elevated extracellular glutamate, resulting from a loss of astrocytic glutamate transport capacity, may contribute to excitotoxic motor neuron (MN) damage in ALS. Accounting for their high excitotoxic vulnerability, MNs possess large numbers of unusual Ca^{2+}-permeable AMPA channels (Ca-AMPA channels), the activation of which triggers mitochondrial Ca^{2+} overload and strong reactive oxygen species (ROS) generation. However, the causes of the astrocytic glutamate transport loss remain unexplained. To assess the role of Ca-AMPA channels on the evolution of pathology *in vivo*, we have examined effects of prolonged intrathecal infusion of the Ca-AMPA channel blocker, 1-naphthyl acetylspermine (NAS), in G93A transgenic rat models of ALS. In wild-type animals, immunoreactivity for the astrocytic glutamate transporter, GLT-1, was particularly strong around ventral horn MNs. However, a marked loss of ventral horn GLT-1 was observed, along with substantial MN damage, prior to onset of symptoms (90–100 days) in the G93A rats. Conversely, labeling with the oxidative marker, nitrotyrosine, was increased in the neuropil surrounding MNs in the transgenic animals. Compared to sham-treated G93A animals, 30-day NAS infusions (starting at 67± 2 days of age) markedly diminished the loss of both MNs and of astrocytic GLT-1 labeling. These observations are compatible with the hypothesis that activation of Ca-AMPA channels on MNs contributes, likely in part through oxidative mechanisms, to loss of glutamate transporter in surrounding astrocytes.

Keywords: Amyotrophic lateral sclerosis; G93A; Excitotoxicity; EAAT2; Motor neuron; 3-Nitrotyrosine; 1-Naphthyl acetylspermine

Introduction

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease characterized by the selective loss of upper and lower motor neurons (MNs). Although the cause of most cases is unknown, observations of deficiencies in glutamate uptake, resulting from a selective loss of the astrocytic glutamate transporter, GLT-1, suggested an excitotoxic contribution (Rothstein et al., 1992, 1995).

Much has been learned about factors that may make MNs particularly susceptible to excitotoxic injury. First, MNs are unusually sensitive to injury mediated through AMPA/kainate type glutamate receptors (Carriedo et al., 1995, 1996; Hugon et al., 1989; Rothstein and Kuncl, 1995), and blockers of these receptors protect MNs from injury caused by prolonged blockade of glutamate uptake (Carriedo et al., 1996; Rothstein et al., 1993).

Likely contributing to this vulnerability, MNs possess substantial numbers of unusual Ca^{2+}-permeable AMPA type glutamate receptor channels (Ca-AMPA channels) (Carriedo et al., 1995, 1996; Van Den Bosch et al., 2000; Vandenberghe et al., 2000). Currently, the best animal models of ALS are provided by rodents harboring mutant forms of the enzyme Cu, Zn superoxide dismutase (SOD1), which are associated with familial ALS in humans. Solidifying the role of Ca-AMPA channels in *in vivo* models of ALS, recent studies indicate that the rate of progression of MN loss in SOD1 mutant mice varies bidirectionally with the level of expression of these channels (Kuner et al., 2005; Tateno et al., 2004; Van Damme et al., 2005).

* Corresponding author. 2101 Gillespie Building, University of California, Irvine, CA 92697-4292, USA. Fax: +1 949 824 1668.
E-mail address: jweiss@uci.edu (J.H. Weiss).

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In addition, Ca-AMPA channels appear to contribute to MN loss in a distinct form of familial ALS, not linked to SOD1 (Lai et al., 2006) and a Ca-AMPA channel blocker was found to be protective in a model of virus induced MN degeneration (Darman et al., 2004).

Mechanisms through which Ca-AMPA channels mediate excitotoxic MN injury are also becoming elucidated. While these channels permit rapid Ca^{2+} entry, MNs buffer cytosolic Ca^{2+} loads poorly (Lips and Keller, 1998), with the consequence that much of the Ca^{2+} is readily taken up into mitochondria, resulting in strong ROS generation (Carriedo et al., 2000; Rao et al., 2003).

However, factors underlying the loss of astrocytic glutamate transport, which likely accounts for excitotoxic MN injury, have been unexplained. Providing a possible clue, recent *in vitro* studies indicated that the ROS produced in MNs in response to Ca-AMPA channel activation was capable of inducing oxidative disruption of glutamate transporters in surrounding astrocytes (Rao et al., 2003). If such a mechanism contributed to glutamate transport disruption in ALS, it could provide the basis for a feed forward cycle that could be integral to disease progression (Rao and Weiss, 2004).

The aim of the present study was to gain insights into ways in which Ca-AMPA channels may contribute to the pathological processes leading to MN degeneration in an *in vivo* animal model of ALS. Specifically, in light of culture studies suggesting that ROS produced in MNs in response to excitotoxic activation might contribute to astrocytic dysfunction, our aim was to examine effects of prolonged Ca-AMPA channel blockade not only on MN loss, but on oxidative changes and transporter levels in surrounding astrocytes as well. As there are presently no Ca-AMPA channel blockers that can be administered systemically, we have carried out prolonged (1 month) intrathecal infusions of the Ca-AMPA channel blocker, 1-naphthyl acetylspermine (NAS) in G93A transgenic SOD1 rats. We find that this treatment slows not only MN loss in these animals, but also slows the loss of GLT-1 glutamate transporter in ventral horn regions near MNs, consistent with the idea that Ca-AMPA channel activation on MNs contributes to the loss of astrocytic glutamate transport.

Materials and methods

Animals

Male hemizygous SOD1 G93A transgenic rats [Tac:N:(SD)-TgN(SOD1G93A)L26H, Emerging Models Program sponsored by Amyotrophic Lateral Sclerosis Association, Taconic laboratories, Germantown, NY] were bred with wild-type females, and offspring genotyped by PCR analysis (Howland et al., 2002); wild-type siblings serve as controls for mutant animals. Animals are killed when they can no longer right themselves within 10 s of being pushed on their side. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Surgical procedure

Intrathecal infusion studies used rats at 65–70 days of age (body weight ~200–300 g). Anesthesia was induced using 5% isoflurane, and maintained at 2.5% at a flow rate of 1 l/min. An incision was made through the skin of the dorsal head and the atlanto-occipital membrane, through which a PE5 catheter was inserted into the subarachnoid space and advanced 6.5–8.5 cm to the lumbar enlargement, as previously described (Hayes et al., 2003). The catheter was connected to an Alzet mini-osmotic pump (model 2004; 200 μl volume, 0.25 μl/h × 30 days) which was pre-filled with NAS (21 mM), or saline, as described (Darman et al., 2004). After surgery, animals were housed individually and body weight recorded daily. In addition, evidence of pain or infection and motor dysfunction were closely monitored. Animals were sacrificed if they appeared distressed or if after 5 days, motor function remained impaired or body weight had not recovered to pre-surgery levels.

Tissue preparation and staining

30 Days after the start of intrathecal infusions, the animals were anesthetized with ketamine, and perfused transcardially with PBS, followed by 4% paraformaldehyde (PFA) for 10 min. The lumbar enlargements of spinal cords were dissected and post-fixed in 4% PFA for 24 h and removed to 30% sucrose/PBS for another 2 days. Serial 25-μm frozen sections were cut from the middle of the lumbar enlargement for ~5 mm in the caudal direction.

After every 10 sections, 3–4 serial sections were set aside for staining. Immunohistochemical stains were carried out on floating sections, blocked (10% FBS, 1 h), and exposed to primary antibody in 10% FBS, 0.3% Triton-X 100 (SMI-32, 1:8000 ip, 1:2000 if, Sternberger Monoclonals, Berkeley, CA; GLT-1, 1:1000, Chemicon, Temecula, CA; 3-nitrotyrosine, 10 μg/ml, Upstate Biotechnology, Waltham, MA). Labeling was visualized either by routine ABC immunoperoxidase techniques or under fluorescence using secondary antibodies linked to fluorophores (Alexafluor 488, Molecular Probes, Eugene, OR; or Cy3, Jackson immunoResearch, West Grove, PA).

Quantification of histopathological changes

Surviving MNs were counted in ventral horn of lumbar spinal slices from each condition (15–20 slices per animal). MNs with pyknotic nuclei, or with a markedly atrophic, irregular or fragmented soma were not counted as alive. For examination of NT and GLT-1 labeling staining in the neuropil surrounding ventral horn MNs, care was taken to ensure that all slices from each experiment were labeled using identical primary and secondary antibody exposures, and fluorescence photographs taken with identical camera settings so that labeling could be compared. For quantification, photographs were imported into an image analysis package (Metamorph software, Molecular Devices Corp., Downington, PA) as 8-bit gray scale images, and regions of neuropil marked surrounding all readily identifiable surviving ventral horn MNs, extending outward from the border of the soma but masking out evident neurons or neuronal processes. For NT, fluorescence intensity was measured in 25-μm zones surrounding identified MNs, and the raw mean intensity value surrounding each MN normalized to that