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Research Report

Marked synergism between mutant SOD1 and glutamate transport inhibition in the induction of motor neuronal degeneration in spinal cord slice cultures

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ABSTRACT

Loss of astrocytic glutamate transport capacity in ALS spinal cord supports an excitotoxic contribution to motor neuron (MN) damage in the disease, and dominant gain of function mutations in Cu/Zn superoxide dismutase (SOD1) cause certain familial forms of ALS. We have used organotypic slice cultures from wild type and G93A SOD1 mutant rat spinal cords to examine interactions between excitotoxicity and the presence of mutant SOD1 in the induction of MN degeneration. Slice cultures were prepared from 1 week old pups, and after an additional week *in vitro*, some were exposed to either a low level (30 μM) of the glutamate uptake inhibitor, *trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) for 3 weeks, or a higher level (50 μM) for 48 h, followed by histochemical labeling to assess MN injury. In wild type animals these exposures caused relatively little MN degeneration. Similarly, little MN degeneration was seen in slices from SOD1 mutant animals that were not exposed to PDC. However, addition of PDC to SOD1 mutant slices resulted in substantial MN injury, which was markedly attenuated by a Ca^{2+} permeable AMPA-type (Ca-AMPA) glutamate channel blocker, or by a nitric oxide synthase antagonist. These observations illustrate the utility of the organotypic culture model for the investigation of intracellular interactions underlying MN degeneration in ALS, and support the hypothesis that activation of Ca-AMPA channels on MNs provides a metabolic burden that synergizes with deleterious effects of mutant SOD1 in the induction of MN injury.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is an adult onset neurodegenerative disease characterized by the selective loss of upper and lower motor neurons (MNs), usually leading to death in 2–5 years. Whereas most cases are sporadic, ~5% are familial (Byrne et al., 2011), with ~20% of familial cases linked to dominant gain of function mutations in the enzyme Cu, Zn superoxide dismutase (SOD1). Rodents harboring mutant forms of this enzyme provide the most widely studied animal models of ALS.

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Abbreviations: Ca-AMPA, Ca^{2+} permeable AMPA; SOD1, Cu/Zn superoxide dismutase; MN, motor neuron; NAS, 1-naphthyl acetylspermine; L-NAME, N-nitro-l-arginine methyl ester; PDC, *trans*-pyrrolidine-2,4-dicarboxylic acid

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In addition to possible diverse genetic contributions, observations of deficiencies in glutamate uptake in human ALS patients, resulting from a selective loss of the astrocytic glutamate transporter, GLT-1, suggested an excitotoxic contribution (Rothstein et al., 1992, 1995). Highlighting the importance of this mechanism, MNs are preferentially injured by prolonged blockade of glutamate uptake (Carriedo et al., 1996; Rothstein et al., 1993). Other studies have revealed factors underlying excitotoxic MN vulnerability.

First, they 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), a feature likely resulting in part from the possession of 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). In addition, 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). Furthermore, this ROS may be able to exit the MN, disrupting glutamate transport in surrounding astrocytes, resulting in increased extracellular glutamate accumulation, and further propagation of the injury cascade (Rao and Weiss, 2004; Rao et al., 2003).

The mechanisms through which SOD1 mutations cause disease are not fully understood; candidate mechanisms include the formation of protein aggregates, and the acquisition of aberrant gain-of-function pro-oxidant effects. Interestingly, mutant SOD1 aggregates adhere to and induce dysfunction of mitochondria in disease affected tissues (Higgins et al., 2002; Liu et al., 2004; Vijayvergiya et al., 2005). In light of evidence that excitotoxic vulnerability of MNs may depend in large part upon mitochondrial Ca^{2+} loading and consequent ROS release (Carriedo et al., 2000; Lips and Keller, 1998; Rao et al., 2003), it seems likely that mitochondria could constitute a locus of interaction of excitotoxic and SOD1 dependent injury mechanisms.

The present study aims to use simplified spinal cord organotypic slice models to study the interaction between the presence of a disease associated SOD1 mutation and excitotoxic stress (induced by prolonged low level glutamate transporter blockade) on MN injury. This model system permits prolonged survival of MNs and good maintenance of their native environment, with preserved interactions with ventral horn astrocytes and local neurons, and thus provides a far more pathophysiologically relevant model of slow MN degeneration than is possible in dissociated cultures. Whereas either mutant SOD1 or low level glutamate uptake blockade alone caused little MN injury, when combined, substantial MN degeneration occurred. In addition, this synergistic injury was substantially attenuated by either a Ca-AMPA blocker or a NOS antagonist, highlighting distinct pathways involved in the injury cascade. This model system may be well suited to the examination of interaction between environmental, physiological and genetic factors in disrupting homeostasis and triggering neurodegenerative cascades in ALS.

2. Results

2.1. *Production and characterization of organotypic spinal cord slice cultures*

We have produced lumbar spinal cord slice cultures from 8 day old rat pups generally according to previously published procedures (Rothstein et al., 1993) (see Experimental procedures). If the cultures appear healthy after 5 days *in vitro*, they generally survive for over a month after plating, often with relatively little MN loss. MNs generally stay in the ventral horn region of spinal cord, which can keep its general shape for a few weeks (but progressively thins out to ~100 μm over 2–3 weeks). MNs in the cultures stain with SMI-32 antibody to non-phosphorylated neurofilaments, which provides excellent staining of MN morphology *in vivo* and in culture (Carriedo et al., 1996). In the slice cultures, double stain with SMI-32 and GFAP shows astrocytes surrounding ventral horn MNs, largely preserving the relationship found *in vivo* between these critical cell types (Fig. 1).

2.2. *Synergism between toxic effects of PDC and the G93A SOD1 mutation in a prolonged exposure model*

To produce the cultures used in these studies, we crossed male hemizygous SOD1 G93A transgenic rats with WT females, and genotyped the offspring (see Experimental procedures) (Howland et al., 2002); slices from WT siblings served as controls for mutant animals. Initial experiments sought to examine the long term survival of MNs in the G93A slices in comparison to WT. Exposures were started one week after plating and continued for three more weeks before staining with SMI-32 and assessing MN survival. Over this time frame, the presence of the G93A mutation caused relatively little detectable effect on either the morphology or number of surviving ventral horn MNs (Fig. 2).

As the mutation alone did not induce significant loss of MNs, we next examined the effect of the glutamate uptake blocker, *trans*-pyrrolidine-2,4-dicarboxylic acid (PDC). This drug acts as a false substrate of the glutamate transporter, and in addition to blocking Na^{+}-dependent glutamate transport, can also facilitate glutamate release via a heteroexchange mechanism (Blitzblau et al., 1996; Volterra et al., 1996). Whereas strong exposures cause relatively rapid glutamate receptor dependent injury resulting from glutamate release, with lower exposures, PDC induces graded dose dependent injury resulting from steady state elevations of extracellular glutamate, similar to that evoked with other glutamate transport blockers. In prior studies 100 μM PDC caused moderate damage to cultured MNs after 24 h (Carriedo et al., 1996), but caused little loss of MNs in organotypic spinal cord cultures similar to those we are using after 15 days (more injury was observed with longer exposures or higher concentrations of PDC) (Matyja et al., 2005; Rothstein et al., 1993). We opted to use a far lower exposure of PDC (30 μM) throughout the 3 week exposure; this also resulted in negligible damage to MNs in wild type slices. However, if the 30 μM PDC was added to the G93A mutant slices, substantial (~80%) MN injury occurred over the ensuing 3 weeks (Fig. 2).