Published in final edited form as:

Brain Res. 2012 April 11; 1448: 153–162. doi:10.1016/j.brainres.2012.02.005.

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 μ M) of the glutamate uptake inhibitor, *trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) for 3 weeks, or a higher level (50 μ M) 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 Ca²⁺ 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.

Keywords

amyotrophic lateral sclerosis; G93A; GLT-1; NOS; excitotoxicity; motor neuron; 1-naphthyl acetylspermine; N-nitro-L-arginine methyl ester

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.,

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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.

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; Rothstein et al., 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; Carriedo et al., 1996; Hugon et al., 1989; Rothstein and Kuncel, 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; Carriedo et al., 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 Materials and Methods). 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 μ m 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 Materials and Methods) (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 hetero-exchange 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 μ M PDC caused moderate damage to cultured MNs after 24h (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 μ M) throughout the 3 week exposure; this also resulted in negligible damage to MNs in wild type slices. However, if the 30 μ M PDC was added to the G93A mutant slices, substantial (~80%) MN injury occurred over the ensuing 3 weeks (Fig. 2).

2.3 Synergistic toxicity can be reproduced in an expedited 48h paradigm

In light of our observation of the marked synergism between mutant SOD1 and glutamate uptake blockade in the induction of MN degeneration in this “prolonged exposure paradigm”, we sought to determine whether similar synergistic injury could be reproduced in a far shorter exposure paradigm, better suited for the study of injury mechanisms and the screening of therapeutic interventions. For this purpose, we mildly modified the media, to one we and others used for brain hippocampal studies (Richichi et al., 2008), the main difference being the addition of a small amount of insulin, which has been reported to aid the maturation of neurons in culture. Neurons in these slice cultures were notably a bit larger and more robust appearing than those used in the prolonged exposure paradigm.

For this “expedited injury” paradigm, PDC exposures were increased to 50 μ M, and slices were processed for immunolabeling 48h after initiation of the exposure. As in the 3 week paradigm, neither the presence of the G93A mutation nor 50 μ M PDC alone induced significant MN degeneration. However, the addition of PDC to transgenic slices again resulted in dramatic potentiation of injury (Fig. 3). Notably, despite the intensity of the exposure, with substantial MN degeneration occurring within 48h, the injury was still quite selective, with little apparent degeneration of dorsal horn neurons (Fig. 4). This pattern of preferential degeneration of ventral horn MNs in SOD1 mutant slice cultures exposed to PDC was quite consistent, and was conspicuously evident in 40 of 46 such slice cultures examined. In contrast, in 20 of 25 SOD1 mutant slice cultures that were not exposed to PDC showed little evidence of damage in either the ventral or dorsal horns.

2.4 Attenuation of injury by a Ca-AMPA channel blocker and by a NOS antagonist

The development of a model system recapitulating relatively selective ventral horn MN degeneration in a fairly short time frame may provide a valuable tool for assessing critical mechanisms in the cell death process and for testing pharmacological interventions. Using the expedited 48 h exposure paradigm, we examined the effects of certain drugs on the MN degeneration. We found that addition of either the Ca-AMPA channel blocker, 1-naphthyl acetylspermine (NAS; 100 μ M), or the nitric oxide synthase antagonist, N-nitro-L-arginine methyl ester (L-NAME; 100 μ M) during the 48h PDC exposure to G93A transgenic slices substantially attenuated consequent MN degeneration (Fig. 3).

3. Discussion

The organotypic slice culture system provides distinct advantages over either dissociated neuronal cultures or *in vivo* animal models for examining events underlying MN degeneration and effects of therapeutic interventions. First, unlike dissociated cultures, slice cultures can survive for weeks, largely preserving the native cellular architecture. This is essential for ALS studies as it is increasingly clear that ALS pathogenesis depends critically upon interactions between ventral horn MNs and their neighboring astrocytes. In addition, unlike animal studies, the slice culture model also enables precise quantitative pharmacological manipulations and direct visualization of the consequences.

In the current studies in the organotypic slice culture paradigm, we find that MNs are able to survive quite well despite either prolonged relatively low level block of the astrocytic glutamate transporters, or the presence of the G93A mutation alone. However, when these perturbations are combined, MN degeneration promptly ensues. Thus the “system” is sufficiently robust to maintain homeostasis in the face of one of these stressors alone, but in combination homeostasis is lost and a degenerative cascade ensues. Furthermore, providing clues to mechanisms of this degenerative cascade, the MN degeneration was substantially attenuated by addition of either a Ca-AMPA channel blocker or a NOS blocker.

3.1 Astrocytic glutamate transport loss and selective MN degeneration in ALS

As discussed above, loss of astrocytic glutamate transport in ALS likely underlies an excitotoxic component to the disease (Rothstein et al., 1992; Rothstein et al., 1995). Furthermore, a number of studies have highlighted factors that may underlie a high excitotoxic vulnerability of MNs; they possess substantial numbers of unusual Ca-AMPA channels (Carriedo et al., 1995; Carriedo et al., 1996; Van Den Bosch et al., 2000; Vandenberghe et al., 2000) but have weak cytosolic Ca^{2+} buffering capacity (Alexianu et al., 1994; Elliott and Snider, 1995; Lips and Keller, 1998; Vanselow and Keller, 2000), such that excitotoxic Ca^{2+} loads are rapidly taken up by mitochondria, resulting in strong mitochondrial ROS generation (Carriedo et al., 2000).

Interestingly, in a dissociated spinal culture model, we have found that the ROS produced in MNs in response to excitotoxic activation appears to be able to leave the MNs, causing loss of glutamate transport function in adjacent astrocytes (Rao et al., 2003). We subsequently examined effects of prolonged intrathecal infusions of the Ca-AMPA channel blocker, NAS, in G93A SOD1 mutant rats, and found that the drug not only slowed MN loss, but also slowed loss of the GLT-1 transporter in astrocytes surrounding the MNs (Yin et al., 2007), suggesting that excitotoxic MN activation contributes to the astrocyte dysfunction. Although the mechanisms underlying astrocyte dysfunction are uncertain, these observations, along with observations by ourselves and others of strong 3-nitrotyrosine labeling in astrocytes surrounding MNs in SOD1 mutant animals (Rao et al., 2003; Yin et al., 2007), led us to suggest that it results in part from effects of ROS produced in mitochondria of MNs that are pathologically overactivated by glutamate. Such a mechanism could provide the basis for a feed forward cascade involving reciprocal interactions between MNs and surrounding astrocytes, in which excitotoxic MN activation results in ROS generation, contributing to loss of astrocytic glutamate transport capacity, resulting in greater excitotoxic MN activation (Rao and Weiss, 2004) (Fig. 5).

3.2 Mutant SOD1 and mitochondria in ALS

The mechanisms through which mutations in SOD1 act to induce MN degeneration are incompletely defined, although most studies have focused on possible gain of function oxidative activities of the enzyme or its tendency to misfold and form aggregates (Estevez et al., 1999; Liu et al., 1998; Valentine and Hart, 2003; Wiedau-Pazos et al., 1996). Irrespective of the precise mechanisms, however, prior studies have provided precedent for synergism between excitotoxic and mutant SOD1 dependent MN injury mechanisms; expression of G93A mutant SOD1 in cultured MNs induced slow MN degeneration that was attenuated by Ca-AMPA channel blockade or by buffering intracellular Ca^{2+} rises (Roy et al., 1998). Furthermore, a number of studies have suggested that mitochondria are a likely locus of these excitotoxicity potentiating effects. Mutant SOD1 interacts with mitochondria (Higgins et al., 2002; Liu et al., 2004; Vijayvergiya et al., 2005), contributing to mitochondrial dysfunction (Mattiazzi et al., 2002; Menzies et al., 2002) and consequent effects including loss of Ca^{2+} homeostasis (Damiano et al., 2006; Jaiswal and Keller, 2009; Tradewell et al., 2011) and increased mitochondrial superoxide production (Ahtoniemi et al., 2008; Zimmerman et al., 2007). Intriguingly, oxidative stress has been reported to cause wild type SOD1 to acquire toxic properties of mutant enzyme (Ezzi et al., 2007), raising the possibility that in an oxidative environment, even wild type SOD1 could associate with and contribute to dysfunction of mitochondria. These observations may provide the basis for a distinct feed forward cascade, occurring within MNs, in which excitotoxic exposures cause oxidative stress, resulting in misfolding of SOD1 and its association with mitochondria. Consequent mitochondrial dysfunction may cause increased MN susceptibility to further excitotoxic exposures (Fig. 5).

3.3 Pharmacological attenuation of the potentiated injury

In the present study, we found the MN injury resulting from exposure of G93A transgenic rat spinal cord slice cultures to 50 μM PDC for 48 h to be substantially attenuated by two drugs. First, the injury was largely prevented by the Ca-AMPA channel blocker, NAS. This was not surprising in light of the large numbers of these channels on MNs and evidence that their activation contributes to MN loss in SOD1 mutant models of ALS.

The other drug that we found to be substantially neuroprotective was the NOS antagonist, L-NAME. In contrast to Ca-AMPA channels, for which a substantial body of data supports their contributions, the evidence for contributions of NOS activation to MN injury in mutant SOD1 linked disease is more mixed.

Arguing against a substantial role in disease, NOS antagonists had little beneficial effect on survival in cultured MNs expressing mutant SOD1 (Doroudchi et al., 2001), and neither NOS antagonists nor removal of the neuronal NOS gene substantially impacted the course of disease in SOD1 mutant animal models (Facchinetti et al., 1999; Upton-Rice et al., 1999). On the other hand, strong increases in 3-nitrotyrosine were found in spinal cord from both human sporadic and SOD1 linked familial ALS, as well as in SOD1 mutant mouse models (Beal et al., 1997; Bruijn et al., 1997; Ferrante et al., 1997). In addition, as discussed above, we have observed prominent 3-nitrotyrosine labeling in astrocytes immediately surrounding MNs in SOD1 mutant animals, consistent with the idea that the reactive species was of MN origin (Rao et al., 2003). Furthermore, glutamate transporters are highly susceptible to oxidative inhibition, and GLT-1 has been specifically shown to be inhibited by oxidative reactions catalyzed by SOD1 mutants (Trotti et al., 1999). Our observations of MN protection by L-NAME suggests that in the simplified and time limited paradigm employed, NOS activation does play a role in the cascade of events resulting in MN degeneration, perhaps by contributing to glutamate transporter dysfunction in astrocytes surrounding MNs, subjecting them to further excitotoxic stress.

3.4 Proposed model and conclusions

Present findings suggest that mutant forms of SOD1, deficiencies in glutamate transporter function, Ca-AMPA channel activation, and NOS activation all interact in the induction of MN degeneration in an organotypic slice model of ALS. How might this occur? As suggested by observations that mutant SOD1 induces mitochondrial dysfunction, promoting ROS generation and Ca^{2+} dyshomeostasis, we propose that the primary relevant site of mutant SOD1 action in this model might be MN mitochondria, increasing the susceptibility of the MNs to excitotoxic injury, mediated in large part via activation of Ca-AMPA channels. We further suggest that, in part due to the effects of mutant SOD1, excitotoxic activation of these MNs may result in excess mitochondrial superoxide production, which may react with NO (generated by NOS) to produce peroxynitrite, possibly accounting for the nitrotyrosine labeling and contributing to glutamate transporter dysfunction in nearby astrocytes. According to this scenario, we would predict that a relevant effect of L-NAME may be to diminish peroxynitrite mediated dysfunction of astrocytic glutamate transporters, thereby helping to maintain transport capacity in a range sufficient for the system to maintain homeostasis.

The present model system may be well suited for the assessment of mechanisms that contribute to pathogenesis in mutant SOD1 linked ALS, as it contains the essential anatomic elements and relationships of the intact spinal cord and enables reproduction of essential features of ALS (including preferential MN degeneration) over a fairly short time period, while permitting precise pharmacological interventions and assessment of consequences. Of note, results in this simplified system may diverge from *in vivo* models, where, for instance, NOS antagonism by itself seems to have little protective efficacy. But in the far more complicated and slowly evolving *in vivo* environment, numerous other events could come into play to obscure this beneficial effect. Might, for instance, NOS antagonists prove to have benefit *in vivo* as components of multi-drug cocktails targetting a set of mechanisms that work together to disrupt the homeostasis needed for MN survival? Simplified systems such as this may be useful for assessing factors affecting this homeostasis, thereby suggesting novel combinations of interventions to test *in vivo*.

4. Experimental Procedures

Animals

Male hemizygous SOD1 G93A transgenic rats [Tac:N:(SD)-TgN(SOD1G93A)L26H, Emerging Models Program sponsored by Amyotrophic Lateral Sclerosis Association, Taconic labs, Germantown, NY] were bred with wild type females, and offspring genotyped by PCR analysis (Howland et al., 2002); wild type (WT) siblings serve as controls for mutant animals. All animal procedures were approved by the Institutional Animal Care and Use Committee.

Slice culture preparation

To prepare slice cultures, 8 day old pups were deeply anesthetized, decapitated and the spinal cords rapidly removed. Transverse sections (400 μ m) were cut from the lumbar spinal cord with a tissue chopper under sterile conditions, and were carefully placed on the surface of Millipore-CM porous (0.4 μ m) membrane inserts (Millipore Corporation, Billerica, MA) which were suspended in 35mm culture wells containing 1 ml of incubation medium. As the slices survive best at an air/fluid interface, any excess medium on the membrane surfaces surrounding the slices was carefully removed. The medium was changed the day after plating and subsequent media exchanges were carried out twice per week. Application of test drugs (PDC, NAS, L-NAME) was via addition to the media, beginning with the 7 day media exchange and continuing through subsequent media exchanges for the duration of the exposure.

For the “prolonged exposure” paradigm, the medium consisted of 50% minimal essential medium (MEM) with 25 mM HEPES; 25% HBSS; 25% heat inactivated horse serum; 2 mM glutamine; 6.4mg/ml D-glucose, pH 7.2. For the “expedited exposure” culture paradigm, the media was altered, with the addition of 1 mM glutamine (total of 3 mM), 3 mM NaHCO₃; 0.5 mM ascorbic Acid, 2 ug/ml insulin, pH 7.2–7.3 (Richichi et al., 2008). The slices are noted to swell within one day of plating, but generally recover after four days. When checked after 5 days *in vitro*, if slices appear shrunken or have irregular edges they are considered damaged and are discarded. Slices that appear intact at this time generally will survive for several more weeks.

After 7 days *in vitro*, the slices were either maintained in media alone or with the addition of drugs (PDC, L-NAME or NAS). After 3 more weeks (for the chronic paradigm) or 48 hours (expedited paradigm), the slices were fixed with 4% PFA, and processed for histological labeling.

Tissue Preparation and staining

Slices subjected to the “prolonged exposure” paradigm were ice protected with 30% sucrose, OCT embedded, and 20 μ m sections cut with a cryostat, prior to immunostaining for the non-phosphorylated neurofilament epitope, SMI-32. The subsectioning permitted better visualization of the smaller and generally faintly staining MNs. In slices subjected to the “expedited exposure” paradigm, neurons generally were generally larger and more robustly labeled, and whole slices were immunostained for SMI-32 (without subsectioning). Of note, these slices thin out during their time *in vitro*, and even in the absence of sub-sectioning all ventral horn MNs in each slice are stained, visualized and counted.

Immunohistochemical stains were carried out on floating sections, blocked (10% HS, 1h), and exposed to primary antibody (SMI-32; Sternberger Monoclonals, Berkeley, CA) in 10% HS, 0.3% Triton-X 100 (1:6000 ip; 1:2000 if). Labeling was visualized either by routine ABC immunoperoxidase techniques or under fluorescence using secondary antibodies

linked to fluorophores (DyLight™488 or DyLight™594, Jackson immunoResearch, West Grove, PA).

Quantification of histopathological changes

Surviving MNs were counted in ventral horn of lumbar spinal slices from each condition, and survival is presented as the % of numbers present in WT slices exposed to media only. Data represent counts from 4–5 independent experiments; with 10–15 slices counted for each condition in each experiment. Care was taken to establish criteria for rating MNs prior to commencing counts, which were carried out by raters blinded to the condition. Only large (>25 µm) distinctly SMI-32 immunoreactive neurons were counted. MNs with pyknotic nuclei, or with a markedly atrophic, irregular or fragmented soma or proximal dendrites were not counted as alive.

Chemicals and Reagents

NAS and L-NAME were purchased from Sigma Chemicals (St. Louis, MO), PDC was purchased from Tocris Bioscience (Park Ellisville, MO), and culture media including HS, MEM and HBSS from Invitrogen (Grand Island, NY). All other chemicals and reagents were obtained from common commercial sources.

Highlights

- Organtypic slice cultures permit prolonged maintenance of spinal cord architecture
- In slice cultures produced from G93A mutant SOD1 rats, MNs survive well
- MNs also survive well in wild type cultures exposed to a glutamate uptake blocker
- In combination, mutant SOD1 and glutamate uptake block cause severe MN injury, which was markedly attenuated by addition of a Ca-AMPA blocker or a NOS blocker
- The model system may be valuable for examination of interactions between cells and mechanisms involved in MN degeneration and testing of therapeutic interventions

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	<i>trans</i> -pyrrolidine-2,4-dicarboxylic acid

Acknowledgments

This work was supported by NIH grant NS36548 (JHW) and a grant from the Muscular Dystrophy Association (JHW).

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Figure 1. Maintained relationship between MNs and surrounding astrocytes in ventral horn of organotypic spinal slice cultures. Spinal cord slice cultures were prepared as described, and maintained *in vitro* for a week before staining. **A:** Low power image shows preserved spinal cord anatomy; SMI-32 labeling shows MNs in ventral horn (on top part of photo). Bar = 500 μm . **B:** Detail from ventral horn showing an SMI-32 labeled MN (red) surrounded by GFAP labeled astrocytes (green). Bar = 50 μm .

Figure 2. Synergistic MN toxicity in organotypic slice cultures: 3 week exposure paradigm. Organotypic slice cultures were prepared as described, and after one week *in vitro*, were subjected to exposures as indicated for 3 weeks. **Top:** Representative fields of SMI-32 labeled ventral horn. Note the relatively preserved MN morphology and numbers in slices from WT animals (alone or with 30 μ M PDC), and in slices from G93A mutant SOD1 animals (Tg), but the marked MN degeneration in the Tg + PDC condition. Arrows indicate representative MNs with clear shrinkage and damaged membranes; arrowhead indicates a MN with fragmented proximal dendrite. Bar = 50 μ m. **Bottom:** Quantification of ventral

horn MN survival. * indicates difference from the WT, 0 PDC condition ANOVA with Student-Newman-Keuls post hoc test ($p < 0.01$).

Figure 3.

Synergistic MN toxicity and neuroprotection in organotypic slice cultures: expedited (48 hour) exposure paradigm. Organotypic slice cultures were prepared as described, and after one week *in vitro*, were subjected to exposures as indicated for 48 hours. **Top:** Representative fields of SMI-32 labeled ventral horn. Note the relatively preserved MN morphology and numbers in slices from WT animals (alone or with 50 μ M PDC), and in slices from G93A mutant SOD1 animals (Tg), but the marked MN degeneration in the Tg + PDC condition. Further note the marked MN protection provided by addition of either NAS (100 μ M) or L-NAME (100 μ M) to the Tg + PDC conditions. Bar = 50 μ m. **Bottom:** Quantification of ventral horn MN survival. & indicates difference from the Tg, 0 PDC

condition, # indicates difference from the WT + PDC condition ($p < 0.001$); * indicates difference from the Tg + PDC condition ($p < 0.02$), by 2 way ANOVA with Student-Newman-Keuls post hoc test.

Figure 4.

Exposure of G93A mutant SOD1 slice cultures to PDC results in relatively selective loss of ventral horn MNs. Organotypic slice cultures were prepared as described from a G93A mutant SOD1 rat pup, and after one week *in vitro*, exposed to media alone for 48 hours (**A**) or with the addition of 50 μ M PDC (the “expedited” injury paradigm, **D**), followed by labeling with SMI-32 to reveal neuronal morphology. Note the good neuronal morphology in both ventral (**B**) and dorsal horns (**C**) upon exposure to media alone, in contrast to the marked degeneration of ventral horn MNs (**E**), and the good preservation of dorsal horn neurons (**F**) upon exposure with PDC. Bar = 500 μ m (**A,D**); or 100 μ m (**B,C, E, F**). The details (**B,C,E,F**) show enlargement of the boxed areas.

Figure 5.

Possible feed forward cascades that may contribute to MN degeneration. (1) Ca-AMPA channels permit rapid Ca^{2+} entry into MNs in response to excitotoxic activation. (2) Since MNs have poor cytosolic Ca^{2+} buffering capacity, large Ca^{2+} loads are buffered by mitochondria. (3) This results in release of excess ROS to the cytosol, and makes MNs particularly susceptible to oxidative stress. (4) Excess ROS produced in MNs may leave the MNs, contributing to dysfunction (including loss of glutamate transport capacity) in nearby astrocytes (mutant SOD1 in the astrocytes may contribute to the dysfunction). The astrocyte dysfunction causes further activation of Ca-AMPA channels (as well as other deleterious effects) on MNs, resulting in further ROS generation. These events may constitute a feed forward cascade occurring between MNs and neighboring astrocytes, leading to increased MN excitotoxic injury. (5) An oxidative environment causes oxidation of mutant (or possibly wild type) SOD1, causing it to misfold, aggregate, and associate with mitochondria, with resultant mitochondrial dysfunction, increased ROS production and impaired mitochondrial Ca^{2+} buffering. These events may constitute a distinct feed forward cascade within MNs resulting in mitochondrial dysfunction with increases in oxidative stress, and an increased vulnerability to excitotoxic injury. (6) Oxidative stress and release of apoptotic factors from damaged mitochondria will both contribute to the eventual MN death.