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Glutamate receptor expression and chronic glutamate toxicity in rat motor cortex

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Abstract

In addition to the loss of spinal motor neurons, amyotrophic lateral sclerosis (ALS) is also associated with degeneration of corticospinal layer V pyramidal neurons and decreased glutamate transport in the cortex. We characterized the glutamate receptors on corticospinal neurons in acutely isolated rat motor cortex slices and found that the synaptic inputs to the corticospinal layer V neurons had a lesser proportional contribution from NMDA receptors relative to AMPA receptors than did layer II/III pyramidal neurons. The synaptic I_{AMPA} was also more inwardly rectified, indicating a greater Ca^{2+} -permeable component, in layer V. In a cortical organotypic slice culture model, blockade of glutamate transporters elevated glutamate in the media and led to pyramidal neuron loss in both layers. The loss of layer V pyramidal neurons was attenuated by antagonists of AMPA/kainate or Ca^{2+} -permeable AMPA receptors, suggesting their therapeutic potential in the protection of the motor cortex in ALS.

I. Introduction

In the motor cortex of amyotrophic lateral sclerosis (ALS) patients, corticospinal layer V pyramidal neurons (upper motor neurons) are progressively lost during the course of the disease. Loss of layer II/III pyramidal neurons also occurs in the severe disease stages (Nihei *et al.*, 1993). Decreased astrocytic glutamate uptake and elevated glutamate concentrations in the cerebrospinal fluid (CSF) suggest that the neuronal toxicity may be mediated by excess glutamate (Rothstein *et al.*, 1990; Rothstein *et al.*, 1992; Rothstein *et al.*, 1995). While the N-methyl-D-aspartate (NMDA) receptor subtype may mediate acute glutamate excitotoxicity of dissociated cortical neurons (Weiss *et al.*, 1990), it is not known whether the non-NMDA or NMDA receptor subtypes contribute to loss of cortical pyramidal neurons in ALS.

Different α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor properties may explain the selective vulnerability of spinal motor neurons to excitotoxicity in ALS (Vandenberghe *et al.*, 2000a; Vandenberghe *et al.*, 2000b; Van Damme *et al.*, 2002; Kawahara *et al.*, 2004). In particular, Ca^{2+} -permeable AMPA receptors, lacking edited GluR2 subunits, have been implicated in some of these studies. It is not known which glutamate receptor subtypes are expressed on upper motor neurons, nor has the role of Ca^{2+} -permeable AMPA receptors in upper motor neuron disease been established. We therefore set out to investigate both the relative proportion of synaptic NMDA versus AMPA receptors and the

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Ca^{2+} permeability properties of synaptic AMPA receptors in upper motor neurons using acute rat brain slices.

For study of disease mechanisms in spinal motor neurons, glutamate transport inhibitors have been successfully used to generate a slow toxicity model in rat spinal cord organotypic cultures (Rothstein *et al.*, 1993) or in vivo, by intrathecal infusion (Hirata *et al.*, 1997). Because it generates long-term low-level glutamate toxicity, blocking glutamate uptake is more likely to resemble the ALS disease process than are acute toxicity models. No reports have described chronic toxicity induced by glutamate transport inhibitors in cortical organotypic cultures. In dissociated mixed neuronal/glial murine cortical cultures, the application of the glutamate uptake inhibitor *trans*-4-carboxy-L-proline (PDC) for up to 24 hours produced neuronal loss without loss of glial cells, that correlated with increased glutamate concentrations and was prevented by NMDA antagonists but not AMPA/kainate antagonists (Velasco *et al.*, 1996).

To specifically study the large layer V pyramidal neurons in tissue maintaining its structural organization, an organotypic culture model of rat motor cortex was developed (Van Westerlaak *et al.*, 2001a). Neuronal death induced by two week treatment with the mitochondrial toxin malonate was inhibited by either NMDA or AMPA/kainate antagonists. Riluzole, a glutamate release inhibitor, was also neuroprotective under these conditions (Van Westerlaak *et al.*, 2001b). However, the effect on cortical pyramidal neurons of chronically elevated glutamate levels is unknown. In the present study, we used patch clamp electrophysiology to characterize glutamate receptor expression in pyramidal neurons in acute cortical slices from young adult rats, and applied an organotypic motor cortex slice culture model to assess the receptor subtypes mediating pyramidal neuron toxicity due to chronically elevated glutamate.

II. Methods

Retrograde labeling of layer V corticospinal neurons

Post-natal day 20 to 25 Sprague-Dawley rats (Harlan, Indianapolis IN) were anesthetized with isoflurane. The cervical spinal cord was exposed and three μL Fluoro-Red dye (Tombow Pencil, Tokyo Japan) was injected into the dorsal spinal cord (Suzuki *et al.*, 2004). After three to seven days post surgery animals were sacrificed and brains were sliced for electrophysiological investigation. The animal protocols have been approved by the University of Chicago Institutional Animal and Care Use Committee.

Electrophysiology

To isolate acute slices, post-natal day 21 to 29 rats were anesthetized with isoflurane and rapidly decapitated into ice cold sucrose slicing buffer containing (in mM): 2.5 KCl, 1 MgCl_2 , 2.5 CaCl_2 , 20 glucose, 1 NaH_2PO_4 , 25 NaHCO_3 , 5 ascorbic acid and sucrose to ~ 315 mOsm. The brain was quickly removed and 250 μm coronal slices were sectioned with a vibratome (Leica, Wetzlar Germany). Slices were incubated in artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 1 MgCl_2 , 2.5 CaCl_2 , 20 glucose, 1 NaH_2PO_4 , and 25 NaHCO_3 , supplemented with 1 mM ascorbic acid, and continuously bubbled with 5% CO_2 /95% O_2 .

Electrodes, 3 to 6 M Ω , were filled with the patch pipette solution (in mM): 114 CsMeSO₄ 6 CsCl, 20 HEPES, 5 EGTA, 3 MgATP, 20 glucose, and 5 QX314 to pH 7.3. Voltage pulses were generated with an EPC-7 patch amplifier (HEKA, Southboro MA) and digitized with an Axon Digidata 1200 series digitizer (Axon, Union City CA). Data were sampled at 10 kHz and filtered at three kHz. Series resistance was 25 Ω M or less during recording. During recording, the extracellular solution was ACSF (without ascorbic acid) with 20 μM bicuculline methiodide (Tocris, Ellisville MO) and 10 μM strychnine to block inhibitory currents. All chemicals were research grade or better from Sigma (St Louis, MO) unless otherwise noted.

Tissue slices were placed on a Zeiss Axioskop2 inverted microscope (Oberkochen Germany). The slice was secured in the recording chamber with a titanium weight. The infrared illumination system (Dage-MTI, Michigan City IN) allowed for direct visual identification of the cells to be patched. Fluorescently labeled neurons were visualized with a fluorescent lamp (AttoArc, Zeiss) and rhodamine filter set (Chroma, Rockingham VT). Layer II/III pyramidal neurons were selected based on somatic size, morphology and were within 300 μm of the pial surface in the primary motor region. The outer edge of fluorescently labeled layer V neurons was typically at least 700–800 μm from the pial surface. Layer V pyramidal neurons were selected based on the presence of the fluorescent label, cortical location, size and shape.

A WECO S-100 stimulator (Winston Electronics, Millbrae CA) was set to a voltage evoking a maximal monosynaptic excitatory post-synaptic current (eEPSC). Neurons were initially held at either -70 mV or $+50$ mV for 600 msec before and two sec after the stimulus. Six traces were averaged from each neuron at each holding potential for subsequent analysis. Peak I_{AMPA} was taken from a one msec window while the cell was held at -70 mV. Holding the cells at $+50$ mV relieves the Mg^{2+} block of the NMDA receptors allowing measurement of I_{NMDA} , which was determined by the current magnitude after AMPA receptors have inactivated in a one msec window 40 msec after the peak I_{AMPA} (determined at -70 mV; see Figure 1). The $I_{\text{NMDA}}/I_{\text{AMPA}}$ is the ratio of I_{NMDA} divided by the peak I_{AMPA} . I_{AMPA} was determined at -70 rather than at $+50$ mV because I_{AMPA} can show rectification depending on the subunit expression patterns (Hollmann *et al.*, 1991; Hume *et al.*, 1991; Verdoorn *et al.*, 1991).

To determine the Ca^{2+} permeability properties of the synaptic AMPA receptors, $10\mu\text{M}$ MK-801 was added to block I_{NMDA} . AMPA receptors lacking the GluR2 subunit and inwardly rectify at positive potentials (Hollmann *et al.*, 1991; Hume *et al.*, 1991; Verdoorn *et al.*, 1991), so that the rectification index (RI) provides a measurement of the contributions of Ca^{2+} -permeable AMPA receptors (Ozawa *et al.*, 1991). RI was determined as the conductance (G) at $+50$ mV divided by G at -70 mV. For each neuron, six eEPSCs were averaged at each holding potential to determine G. G at $+50$ mV and at -70 mV were determined from a one msec isochronal window, set to the peak current at -70 mV. The E_{rev} is considered to be 0 mV given the high Na^+ recording conditions. The range of E_{rev} from the literature with similar recording conditions is -4.0 to 1.25 mV (Iino *et al.*, 1990; Lerma *et al.*, 1994; Koh *et al.*, 1995; Isa *et al.*, 1996).

Organotypic Tissue Culture

Post-natal day 2 rat pups were anesthetized with isoflurane, rapidly decapitated and dissected in Gey's Balanced Salt Solution supplemented with glucose to a total of 5.4 mg/mL. Using a McIlwain tissue chopper (Mickle Laboratory Engineering Co, Surrey England), 400 μm thick organotypic slices were taken from the frontal cortex where the striatum is present (Van Westerlaak *et al.*, 2001a). Three slices per well were grown on a Millicell-CM filter (Millipore, Billerica MA) in 1.2 mLs of culture media containing 25% horse serum, 25% Hanks' Balanced Salt Solution, 50% MEM, supplemented glucose to a total of 5.4 mg/mL, 2 mM L-glutamine, 1 $\mu\text{g/mL}$ amphotericin B and 100 IU/mL penicillin/streptomycin (Invitrogen, Carlsbad CA). Cultures were fed twice per week by exchanging half of the media in the well.

Toxicity assays began after two weeks in culture. 250 μM PDC (Tocris) was applied alone or in combination with 10 μM 6,7-dinitroquinoxaline-2,3-dione (DNQX, Tocris), 250 nM philanthotoxin-343 (Phtx) or 840 nM ketamine (Fisher Scientific, Hampton NH). Slices were fixed, stained and evaluated following the guidelines of Van Westerlaak *et al.* (2001a and 2001b), with these subsequent modifications. A non-phosphorylated neurofilament (NF) antibody (NF-M+H, catalog number 13–1300, Zymed/Invitrogen, Carlsbad CA) was used at 1:85 to label pyramidal neurons. NF(+) neurons include corticospinal pyramidal neurons and

other pyramidal neurons in layers II/III and V (Voelker, et al. 2004). The secondary antibody was Cy5-goat anti-mouse 1:500 (Jackson ImmunoResearch, West Grove PA). Slices were wet mounted onto glass slides. Photomicrographs of the primary motor region of dorsal cortex were evaluated using ImageJ software by an investigator blinded to the conditions. Layer II/III was considered within 300 μ m of the pial surface. Layer V pyramidal neurons were identified as NF+ neurons with large cell bodies near the striatum. Neuronal density in each layer was calculated as NF+ pyramidal neurons divided by the area of cortical layer encompassed.

Glutamate Assay

Glutamate concentrations in the culture media were determined using the Amplex Red Glutamic Acid/Glutamate Oxidase kit (Invitrogen) according to manufacturer's instructions.

III. Results

Corticospinal layer V pyramidal neurons have less synaptic NMDA current relative to layer II/III neurons

Layer V corticospinal neurons, retrogradely labeled with dorsal spinal cord injection of Fluoro-Red dye as previously described (Suzuki *et al.*, 2004). The corticospinal neurons were easily visualized as a band of fluorophore-containing neuronal cell bodies (Figure 1A; Swanson, 2003). The dye had a punctuate distribution in the cell bodies of pyramidal neurons (Figure 1B). For the electrophysiology experiments, each layer V pyramidal neuron was identified by size, shape and the presence of dye. Pyramidal neurons of cortical layer II/III, identified by morphology and location within 300 μ m of the pial surface, were also examined as a comparison group.

The glutamate receptor subtypes that contributed to the eEPSCs were assessed by whole cell recordings from layer II/III and corticospinal layer V pyramidal neurons. The non-NMDA-mediated currents are likely mediated by AMPA receptors because kainate receptors on cortical layer V pyramidal neurons are located extrasynaptically (see discussion; Ali, 2003; Eder *et al.*, 2003). eEPSCs recorded at -70 mV reflect AMPA receptor activation because NMDA receptors are blocked by Mg^{2+} at this potential. Therefore, the I_{AMPA} peak was determined as the peak inward current at -70 mV. With the Mg^{2+} block relieved at $+50$ mV, the eEPSC is a mixture of I_{AMPA} and I_{NMDA} . The magnitude of I_{AMPA} at $+50$ mV reflects both the number of receptors activated and the rectification of the AMPA receptors. Receptors lacking the GluR2 subunit are Ca^{2+} -permeable, inwardly rectify, and generate less current at $+50$ mV (Hollmann *et al.*, 1991; Hume *et al.*, 1991; Verdoorn *et al.*, 1991). I_{AMPA} inactivates faster than I_{NMDA} , decaying to baseline in less than 40 msec (Figure 2A and B). Thus, at $+50$ mV the outward current remaining 40 msec after the peak I_{AMPA} was taken to represent I_{NMDA} (Figure 2A and B; see also Myme *et al.*, 2003). I_{NMDA} at $+50$ mV divided by the peak I_{AMPA} at -70 mV provided a relative measure of I_{NMDA} and I_{AMPA} . I_{AMPA} was chosen as the denominator because two of the synapses from layer V pyramidal neurons had little, if any, synaptic I_{NMDA} . The average I_{NMDA}/I_{AMPA} for retrograde labeled layer V neurons was 0.5 ± 0.1 , significantly less than the average of 1.3 ± 0.1 for layer II/III pyramidal neurons (Figure 2C, $n=18$ for each layer, $*p<0.001$).

This difference in I_{NMDA}/I_{AMPA} , a relative measure of the contribution of the glutamate receptor subtypes to the synaptic current, might arise from less I_{NMDA} or greater I_{AMPA} in corticospinal layer V pyramidal neurons. The stimulation applied was set to obtain maximal monosynaptic input for each individual pyramidal neuron. With this method, I_{AMPA} at -70 mV was not different between pyramidal neurons in layer II/III and corticospinal layer V pyramidal neurons. The average synaptic I_{NMDA} , however, was significantly less in the layer V neurons (Figure 2D, $*p<0.001$; $n=18$ for each layer). In one neuron there was no detectable

synaptic I_{NMDA} , and in a second cell $I_{\text{NMDA}}/I_{\text{AMPA}}$ was less than 0.01. Overall, the data suggested a lesser proportional contribution of NMDA receptors to synaptic responses in layer V than in layer II/III pyramidal neurons.

Synapses onto corticospinal layer V pyramidal neurons include more Ca^{2+} -permeable AMPA receptors than do those in layer II/III pyramidal neurons

To characterize the Ca^{2+} permeability of the synaptic AMPA receptors, AMPA-mediated eEPSCs were isolated by perfusing the tissue slices with 10 μM MK-801 to block I_{NMDA} . The remaining current was AMPA receptor-mediated and was blocked by 10 μM DNQX (Supplementary Figure 1 and Discussion). Representative averaged traces from layers II/III and V before (solid line) and after (dotted line) exposure to 10 μM MK-801 (Figure 3A and B) showed that the I_{AMPA} amplitude varied at +50 mV. The degree of Ca^{2+} -permeability of the synapses is reflected in the rectification index (RI), which is $G_{+50\text{mV}}/G_{-70\text{mV}}$ measured from the peak -70mV I_{AMPA} and +50 mV I_{AMPA} in the same isochronal window (Figure 3A & 3B, long dashed line). Synapses with more inwardly rectifying Ca^{2+} -permeable AMPA receptors have a lower RI measurement. In contrast, Ca^{2+} -impermeable AMPA receptors do not rectify and the RI is closer to unity. Synapses onto layer V pyramidal neurons had a lower RI (0.6 ± 0.1 , $n=9$) than those onto layer II/III (1.0 ± 0.1 ; $n=9$; $*p < 0.02$) indicating a greater contribution from Ca^{2+} -permeable AMPA receptors in layer V (Figure 3C). Together, these data support the idea that synaptic inputs to corticospinal layer V pyramidal neurons had relatively less I_{NMDA} and more Ca^{2+} -permeable I_{AMPA} than the pyramidal neurons in layer II/III.

In the hippocampus, mossy fiber-interneuron synapses that have more Ca^{2+} -permeable AMPA receptors, represented by a lower RI, tend to have less I_{NMDA} (Lei & McBain, 2002). To examine this issue in cortical neurons, we compared the $I_{\text{NMDA}}/I_{\text{AMPA}}$ under control conditions and RI after MK-801 application in the neurons in which both measures were made. Comparison of $I_{\text{NMDA}}/I_{\text{AMPA}}$ and RI across all of the pyramidal neurons revealed a significant correlation (Figure 3D, $n=8$ for each layer, $r=0.757$). At synapses where the $I_{\text{NMDA}}/I_{\text{AMPA}}$ was the lowest, the RI was the lowest, signifying a greater contribution of Ca^{2+} -permeable AMPA receptors to synapses with a lower density of NMDA receptors.

Long-term organotypic cultures maintain cortical organization

To examine the role of glutamate receptor subtypes in chronic excitotoxicity, we established an organotypic culture model of the rat motor cortex similar to previous reports (Van Westerlaak *et al.*, 2001a; Van Westerlaak *et al.*, 2001b). The cortical organization was maintained up to four weeks in culture and pyramidal neurons were easily counted (Figure 4A). To identify surviving pyramidal neurons in these organotypic slices, we used a mouse monoclonal antibody against non-phosphorylated neurofilament M+H. It labeled layer II/III pyramidal neurons and corticospinal layer V pyramidal neurons; reportedly layer V pyramidal neurons that project to the contralateral cortex are not labeled by this antibody (Voelker *et al.*, 2004). We confirmed the ability of this antibody to label layer II/III pyramidal neurons and corticospinal neurons (Supplementary Figure 2).

PDC treatment raises glutamate levels in the culture medium

As both NMDA and AMPA receptors are present at synapses onto pyramidal neurons, either or both subtypes may be involved in neurotoxicity resulting from increased extracellular glutamate. Slowly elevating glutamate concentrations, over the course of two weeks, better represents the chronic toxicity that occurs in ALS than does an acute 24 hour applications. Cortical organotypic cultures from post-natal day two rat pups were grown for 14 days, and then the glutamate uptake inhibitor PDC (250 μM) was applied. Glutamate concentrations increased from ~ 200 nM to 982 nM and 1.52 μM after 7 and 14 days of treatment, respectively

(Figure 4B, $n=4-5$, $*p<0.05$). The presence of the antagonists 10 μM DNQX (AMPA/kainate antagonist), 250 nM Phtx (a Ca^{2+} -permeable AMPA receptor antagonist) or 840 nM ketamine (NMDA receptor antagonist) alone did not alter the glutamate concentration in the culture media after 28 days in culture, nor did addition of the antagonists with 250 μM PDC alter the glutamate concentration relative to PDC alone (Supplementary Figure 3).

PDC treatment causes a loss of pyramidal neurons in organotypic cultures

To determine the toxicity of chronically elevating glutamate using PDC for two weeks, NF immunoreactive pyramidal neurons were counted by an investigator blinded to the treatment conditions. In both layer II/III and layer V, 250 μM PDC produced near-complete loss of pyramidal neurons after two weeks (Figure 4C, 4D). At a lower concentration of 100 μM , PDC produced partial loss in both layers (not shown). To determine the glutamate receptor subtypes contributing to the chronic excitotoxic death, DNQX (10 μM), Phtx (250 nM), or ketamine (840 nM) were added alone, or with the addition of PDC, for a two week exposure. Neither DNQX nor Phtx alone altered neuronal survival. In both layer V and layer II/III, the AMPA/kainate antagonist DNQX produced near complete protection against PDC-induced pyramidal neurons loss (Figure 4, $p<0.05$ by post-hoc Student-Newman Keuls testing). Interestingly, the Ca^{2+} -permeable AMPA antagonist Phtx produced strong protection of layer V pyramidal neurons, while in layer II/III any protective effect of Phtx failed to reach statistical significance. The application of the NMDA antagonist ketamine (840 nM) with PDC also failed to produce significant protection in either layer compared to PDC application alone. However, ketamine alone significantly reduced cell survival, compared to controls (Figure 4, $n=5-8$ replicates, two-way ANOVA with Student-Newman-Keuls post-hoc test, $p<0.05$) as did other NMDA antagonists (1 μM MK-801 or 1 μM DL-AP5, data not shown), consistent with prior reports of NMDA antagonist toxicity (Fix *et al.*, 1993; Jevtovic-Todorovic *et al.*, 2001). This inherent toxicity of NMDA antagonists prevented reliable assessment of NMDA receptor contributions to PDC-induced pyramidal cell loss. These data indicate that the pyramidal neurons of both cortical layers V and II/III were sensitive to the mildly elevated glutamate concentrations induced by PDC, and that receptors of the AMPA/kainate class played an important role in mediating this toxicity. Chronic glutamate excitotoxicity in layer V pyramidal neurons, in particular, was inhibited by an antagonist of Ca^{2+} -permeable AMPA receptors.

IV. Discussion

This study is the first to specifically characterize synaptic glutamate receptors expressed in identified corticospinal neurons and layer II/III pyramidal neurons. It is also the first study to examine the contribution of these receptors to chronic glutamate toxicity. We found significant differences in the NMDA versus AMPA receptor expression patterns and in the Ca^{2+} permeability of AMPA receptors at the synapses onto upper motor neurons as compared to layer II/III pyramidal neurons. In an organotypic culture model incorporating chronic excitotoxicity, antagonists of AMPA/kainate or Ca^{2+} -permeable AMPA receptors were protective of layer V pyramidal neurons.

Synaptic I_{NMDA} and I_{AMPA} at pyramidal neurons differ in layer II/III and layer V

Previous reports indicated $I_{\text{NMDA}}/I_{\text{AMPA}}$ ratios of 1.25 from layer II/III of prefrontal and visual cortex (Myme *et al.*, 2003) and 1.8 from layer V of the agranular frontal cortex (Kumar & Huguenard, 2001). These numbers are close to that measured in layer II/III in this report (1.3 ± 0.1) but differ from our $I_{\text{NMDA}}/I_{\text{AMPA}}$ in layer V (0.5 ± 0.1). A reason for the difference in layer V $I_{\text{NMDA}}/I_{\text{AMPA}}$ ratios may be due to the specialized nature of pyramidal neurons that project to the spinal cord versus those in the agranular frontal cortex. Some synapses onto the upper motor neurons clearly had little NMDA current (Figure 2).

Our experiments identified more synaptic Ca^{2+} -permeable AMPA receptors on corticospinal neurons than on the layer II/III pyramidal neurons. In an organotypic culture of the prefrontal cortex from post-natal day 8–12 rats at two days in culture, synaptic AMPA receptors recorded from pyramidal neurons in layers II/III to V also had a linear current-voltage relationship (Beique & Andrade, 2003). This is consistent with the RI from our layer II/III pyramidal neurons. Contrasting with our findings in corticospinal neurons, layer V pyramidal neurons in the primary visual cortex reportedly had a low Ca^{2+} -permeability of AMPA receptors when the reversal potential was measured by a fast application of glutamate to outside out patches ($P_{\text{Ca}^{2+}}/P_{\text{K}} = 0.05$, Jonas *et al.*, 1994). The relatively large contribution of Ca^{2+} -permeable AMPA receptors may be a unique property of synapses at corticospinal neurons.

Neurotoxicity in organotypic cortical cultures in response to chronically elevated glutamate

Exposure of organotypic slice cultures to the glutamate uptake inhibitor PDC for two weeks elevated glutamate concentrations in the media and caused a loss of pyramidal neurons providing a model resembling the ALS disease process. The organotypic culture model, while offering a chance to test the role of different glutamate receptor subtypes in chronic glutamate-induced toxicity, involved some limitations. First, our experiments quantified survival of layer V pyramidal neurons identified by morphology and immunostaining, but not by retrograde labeling establishing projection to the spinal cord, as in the electrophysiology experiments. It is possible that these counts included some non-corticospinal neurons, potentially diluting the effects specific for corticospinal neurons and preventing detection of preferential injury to this subset. However, in the dorsal portion of cortex that was studied, the majority of large pyramidal neurons of layer V project to the cervical spinal cord (see Figure 1), making it likely that our survival data for layer V pyramidal neurons is representative of the properties of the corticospinal neurons. It is also possible that glutamate receptor expression pattern develop differently in corticospinal neurons in the organotypic cultures as compared to *in vivo*. Ultimately, confirmation of these findings inferred from the organotypic culture model will require *in vivo* studies. Despite these limitations, the toxicity studies in organotypic cultures offered important insights into mechanisms of neurodegeneration induced by chronic low-level glutamate exposures.

The glutamate concentrations in the media from our organotypic culture controls are identical to the 176 nM glutamate reported in the CSF of control human subjects (Spreux-Varoquaux *et al.*, 2002). With PDC treatment, the glutamate concentrations in culture increase to the range of CSF glutamate levels reported in a subset of ALS patients of 80 nM to 1.43 μM (Spreux-Varoquaux *et al.*, 2002). Another study reports that glutamate concentrations in CSF exceeded 10 μM in 39% of ALS patients (Shaw *et al.*, 1995), which is well above the amount of glutamate needed in organotypic cultures to cause toxicity. Glutamate measured in the media represents a global increase that would activate synaptic and extrasynaptic glutamate receptors to affect neuronal viability. Both synaptic and extrasynaptic NMDA receptors are capable of mediating neurotoxicity (Sattler *et al.*, 2000), and both may be contributing to the toxicity observed in the present model. While the observed toxicity of chronic glutamate elevation was not selective for layer V neurons over those of layer II/III, involvement of pyramidal neurons of both cortical layers is consistent with described pathological findings of late ALS (Nihei *et al.*, 1993).

Previous studies of pyramidal neuron loss in cortical organotypic cultures used mitochondrial inhibition to produce toxicity (Van Westerlaak *et al.*, 2001a; Van Westerlaak *et al.*, 2001b). This toxicity was prevented by an NMDA antagonist, an AMPA/kainate antagonist or a glutamate release inhibitor. Our present study of the toxicity of PDC-induced glutamate elevations has also found a necessary role for AMPA/kainate receptors in chronic toxicity in layer V pyramidal neurons, and has suggested a novel role of Ca^{2+} -permeable AMPA receptors in this chronic excitotoxicity. In either cortical layer, the co-application of DNQX with PDC

restored pyramidal cell survival to control levels, indicating an important contribution of AMPA/kainate receptors to the chronic toxicity associated with low level glutamate accumulation. As ketamine and other NMDA receptor antagonists alone produced neuronal toxicity with the chronic two week exposures, consistent with previous reports of toxic effects of chronic NMDA receptor blockade (Fix *et al.*, 1993; Jevtovic-Todorovic *et al.*, 2001), definitive testing of the contributions of NMDA receptors to the chronic glutamate toxicity in the pyramidal neurons was impossible. Importantly, in layer V pyramidal neurons, Phtx, a selective antagonist of Ca^{2+} -permeable AMPA/kainate receptors, also produced significant and substantial protection against chronic PDC-induced toxicity, while not producing statistically significant protection in layer II/III neurons. This result, concordant with the greater contribution of Ca^{2+} -permeable AMPA receptors to synaptic responses in layer V corticospinal neurons, suggests that this sub-class of receptors has a necessary role in the chronic excitotoxicity induced by blockade of glutamate transport, and that they may be a viable therapeutic target for protection of the corticospinal neurons.

AMPA or kainate receptors?

This paper addresses the role of NMDA and non-NMDA (AMPA or kainate) receptors on the post-synaptic membranes of corticospinal layer V pyramidal neurons and pyramidal neurons in layer II/III. The electrophysiology and toxicity studies used DNQX, which blocks both AMPA and kainate receptors. Kainate receptors on cortical pyramidal neurons have been identified extrasynaptically (Ali, 2003; Eder *et al.*, 2003). When synaptic kainate-mediated currents were present on cortical pyramidal neurons, they accounted for only 10% of the total AMPA/kainate current (Ali, 2003; Eder *et al.*, 2003). Thus, the evidence suggests that the vast majority of the synaptic current present at -70 mV in our electrophysiological studies was dependent on AMPA receptors. However, in the toxicity experiments in the present study, the protection produced by DNQX or Phtx does not distinguish pharmacologically between AMPA and kainate-specific receptors. A potential role for kainate receptors in acute 24-hour glutamate toxicity has been reported in cerebellar granule neuron cultures (Carroll *et al.*, 1998), and in a small subpopulation of hippocampal neurons, in which a 30 minute exposure to toxin produced kainate receptor-mediated toxicity after 24 hours (Silva *et al.*, 2001). Though the more prevalent postsynaptic expression of AMPA receptors than kainate receptors makes AMPA receptors the likely candidate for mediating chronic glutamate toxicity, further studies are needed to fully distinguish the role of AMPA versus kainate receptors in the organotypic cortical culture model of chronic glutamate excitotoxicity.

Summary

Corticospinal layer V pyramidal neurons from post-natal day 21 to 29 rat motor cortex express relatively less synaptic I_{NMDA} than pyramidal neurons in layer II/III, and also express more Ca^{2+} -permeable AMPA receptors at synapses, with an inverse correlation between synaptic expression of NMDA receptors and of Ca^{2+} -permeable AMPA receptors. In an organotypic model in which chronically elevated glutamate levels mediate neurotoxicity, antagonists of AMPA/kainate receptors and in particular of Ca^{2+} -permeable AMPA receptors prevented layer V pyramidal neuron loss. The neuroprotection achieved by blocking AMPA/kainate receptors, and specifically the Ca^{2+} -permeable subclass of these receptors, suggests that selective antagonist block of chronic excitotoxicity induced by low levels of glutamate is a potential therapeutic approach for limiting the corticospinal neuron loss in ALS.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Retrograde labeling of corticospinal neurons

Fluoro-Red dye was injected into the spinal cord 7 days prior to imaging. A) Coronal slices from a post-natal day 28 animal show that layer V upper motor neurons are labeled. B) This method labels large pyramidal neurons consistent with their identification as corticospinal neurons.

Figure 2. NMDA vs. AMPA currents in layer II/III & V pyramidal neurons

Representative averaged trace of 6 evoked EPSCs at -70 mV and $+50$ mV from layer II/III (A) and retrograde labeled layer V (B) pyramidal neurons. I_{AMPA} is measured at the peak of the -70 mV trace. I_{NMDA} is measured 40 msec after the peak I_{AMPA} from the $+50$ mV trace (dashed line). C) Scatter plot and bar graph of the $I_{\text{NMDA}}/I_{\text{AMPA}}$ ratio for layer II/III and layer V pyramidal neurons. $I_{\text{NMDA}}/I_{\text{AMPA}}$ is significantly less in layer V pyramidal neurons (mean $\pm$ sem shown; $n=18$ for each layer, $p<0.001$, Student's t -test). D) I_{AMPA} and I_{NMDA} for pyramidal neurons in layer II/III or layer V (mean $\pm$ sem; $*p<0.001$, Student's t -test).

Figure 3. AMPA rectification of layer II/III & V pyramidal neurons

Representative average trace of 6 evoked EPSCs at -70 mV and $+50$ mV from layer II/III (A) and retrograde labeled layer V (B) pyramidal neurons before (solid line) and after (dotted line) 10μ M MK-801 application. Rectification index (RI) is $G_{+50 \text{ mV}}/G_{-70 \text{ mV}}$ measured at the peak I_{AMPA} after MK-801 application (long dashed line). C) Scatter plot and bar graph (mean $\pm$ sem) of the RI for layer II/III (n=9) and layer V (n=9) pyramidal neurons, (* $p < 0.02$, Student's t-test). D) Linear correlation between I_{NMDA}/I_{AMPA} and RI for individual pyramidal neurons in layer V (white circles, n=8) and layer II/III (filled circles, n=8), $r = 0.757$.

Figure 4. PDC-induced glutamate accumulation and pyramidal neurons loss in organotypic cultures

A) Cortical organotypic slice cultures. Immunostaining with a nonphosphorylated NF M+H antibody labeling pyramidal neurons shows maintained cortical organization after 3 weeks in culture. Arrows: representative layer V and layer II/III pyramidal neurons. B) Time-dependent increase of glutamate concentrations in the culture media in the presence 250 μM PDC (mean $\pm$ sem; $n=4-7$, * $p<0.05$ vs. control, one-way ANOVA with Student-Newman-Keuls post-hoc testing). C), D) Blinded counts (mean $\pm$ sem, $n=5-8$ replicates for each condition) of NF-staining pyramidal neuron cell bodies in layers II/III (C) and layer V (D) with control media, 250 μM PDC, or 10 μM DNQX, 250 nM Phtx or 840 nM ketamine with or without 250 μM PDC. In layer II/III, only DNQX prevented the severe pyramidal neuron loss produced by PDC, while ketamine alone was toxic, and failed to produce protection reaching statistical significance (two-way ANOVA, PDC treatment significant at $p<0.001$ and antagonist treatment significant at $p<0.01$; *different from control and #different from PDC alone by Student-Newman-Keuls post-hoc testing, $p<0.05$). In layer V, both DNQX and Phtx significantly protected against the severe pyramidal neuron loss produced by PDC. Again, ketamine alone was toxic, and failed to produce protection reaching statistical significance (two-way ANOVA, PDC treatment significant at $p<0.001$ and antagonist treatment significant

at $p < 0.001$; *different from control and #different from PDC alone by Student-Newman-Keuls post-hoc testing, $p < 0.05$).