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TNF- α triggers rapid membrane insertion of Ca²⁺ permeable AMPA receptors into adult motor neurons and enhances their susceptibility to slow excitotoxic injury

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Abstract

Excitotoxicity (caused by over-activation of glutamate receptors) and inflammation both contribute to motor neuron (MN) damage in amyotrophic lateral sclerosis (ALS) and other diseases of the spinal cord. Microglial and astrocytic activation in these conditions results in release of inflammatory mediators, including the cytokine, tumor necrosis factor- α (TNF- α). TNF- α has complex effects on neurons, one of which is to trigger rapid membrane insertion of α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) type glutamate receptors, and in some cases, specific insertion of GluA2 lacking, Ca²⁺ permeable AMPA receptors (Ca-perm AMPAR). In the present study, we use a histochemical stain based upon kainate stimulated uptake of cobalt ions (“Co²⁺ labeling”) to provide the first direct demonstration of the presence of substantial numbers of Ca-perm AMPAR in ventral horn MNs of adult rats under basal conditions. We further find that TNF- α exposure causes a rapid increase in the numbers of these receptors, via a phosphatidylinositol 3 kinase (PI3K) and protein kinase A (PKA) dependent mechanism. Finally, to assess the relevance of TNF- α to slow excitotoxic MN injury, we made use of organotypic spinal cord slice cultures. Co²⁺ labeling revealed that MNs in these cultures possess Ca-perm AMPAR. Addition of either a low level of TNF- α , or of the glutamate uptake blocker, *trans*-pyrrolidine-2,4-dicarboxylic acid (PDC) to the cultures for 48 h resulted in little MN injury. However, when combined, TNF- α +PDC caused considerable MN degeneration, which was blocked by the AMPA/kainate receptor blocker, 2,3-Dihydroxy-6-nitro-7-sulfamoylbenzo (F) quinoxaline (NBQX), or the Ca-perm AMPAR selective blocker, 1-naphthyl acetylspermine (NASPM). Thus, these data support the idea that prolonged TNF- α elevation, as may be induced by glial activation, acts in part by increasing the numbers of Ca-perm AMPAR on MNs to enhance injurious excitotoxic effects of deficient astrocytic glutamate transport.

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Keywords

tumor necrosis factor-alpha; amyotrophic lateral sclerosis; ALS; AMPA; Ca²⁺ permeable AMPA receptors; slice culture; motor neuron; protein kinase A; phosphatidylinositol 3 kinase

Introduction

Amyotrophic lateral sclerosis (ALS) is a catastrophic disease characterized by relatively selective degeneration of upper and lower (spinal) motor neurons (MNs), for which there is as yet no good treatment. Observations of deficiencies in spinal cord glutamate uptake in human ALS patients, resulting from selective loss of the astrocytic glutamate transporter, GLT-1, suggested an excitotoxic contribution (Rothstein et al., 1992; Rothstein et al., 1995). It is progressively evident that cell death in ALS depends critically upon interactions between MNs and neighboring cells. Activation of both microglia and astrocytes occur prominently in both human disease and animal models of ALS (Philips and Robberecht, 2011; Wegorzewska et al., 2009), likely contributing to MN injury via a number of mechanisms, a key one of which may be release of factors including the cytokine tumor necrosis factor-alpha (TNF- α) (Mhatre et al., 2004; Tweedie et al., 2007).

Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors are tetramers, comprised of combinations of subunits (GluA1-4). Most AMPA receptors are Ca²⁺ impermeable due to the presence of a GluA2 subunit; GluA2 lacking receptors are Ca²⁺ permeable. TNF- α has complex effects, which could be either beneficial or injury promoting (Cheng et al., 1994; Ghezzi and Mennini, 2001; Hermann et al., 2001; Pickering et al., 2005). One injury promoting effect is the rapid insertion of AMPA type glutamate receptors into the plasma membrane (Beattie et al., 2002; Hermann et al., 2001; Stellwagen and Malenka, 2006; Yu et al., 2002). Interestingly, the majority of these AMPA receptors contain GluA1 (Beattie et al., 2002; Leonoudakis et al., 2008; Ogoshi et al., 2005; Stellwagen et al., 2005). Furthermore, although some studies showed membrane increases in GluA2 as well, TNF- α preferentially caused the rapid insertion of GluA2 lacking, Ca²⁺-permeable AMPA receptors (Ca-perm AMPAR) in hippocampal pyramidal neurons (which normally have relatively low levels of these receptors) (Ogoshi et al., 2005; Stellwagen et al., 2005) and spinal dorsal horn neurons (Choi et al., 2010), resulting in increased susceptibility to excitotoxic injury (Leonoudakis et al., 2008).

Embryonic spinal MNs possess significant numbers of Ca-perm AMPAR under basal conditions (Carriedo et al., 1995; Carriedo et al., 1996; Van Den Bosch et al., 2000; Vandenberghe et al., 2000), there is indirect evidence that adult MNs possess them as well (Corona and Tapia, 2007; Darman et al., 2004; Tateno et al., 2004; Williams et al., 1997; Yin et al., 2007), and it is likely that their presence plays a role in the high susceptibility of MNs to excitotoxic injury. Past studies concerning effects of TNF- α on MNs, and consequences for disease have mixed results. One recent study found spinal cord trauma to cause a rapid appearance of Ca-perm AMPAR on neurons via a TNF- α dependent mechanism, which contributed to the resultant injury (Ferguson et al., 2008). However, another study reported TNF- α to reduce numbers of Ca-perm AMPAR on cultured MNs (Rainey-Smith et al., 2010). Regarding possible roles in models of ALS, TNF- α appeared to contribute to MN loss in the wobbler mouse model (Bigini et al., 2008), but knock out of the TNF- α gene had no effect on disease progression in superoxide dismutase type 1 (SOD1) mutant mouse ALS models (Gowing et al., 2006).

The present study employs a histochemical stain based upon kainate-stimulated uptake of Co²⁺ ions ("Co²⁺ labeling") to confirm the presence of functional Ca-perm AMPAR on adult

ventral horn MNs in lumbar spinal cord slices, and examine effects of TNF- α on their numbers. We further make use of organotypic spinal cord slice cultures to determine effects of low levels of TNF- α on slow excitotoxic MN degeneration caused by subtoxic exposures to the glutamate uptake blocker, *trans*-pyrrolidine-2,4-dicarboxylic acid (PDC). Present results may be relevant to ALS or other conditions in which slow excitotoxicity and inflammation contribute to MN damage.

Materials and Methods

Animal tissue collection and handling

All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animal and approved by the University of California Irvine Institutional Care and Use committee.

Acute spinal cord slice preparation—Female Sprague Dawley rats (55–65 day old, 175–250g, purchased from Charles River Laboratories, Wilmington, MA) were deeply anesthetized with 1% sodium pentobarbital (40 mg/kg), decapitated, lumbar laminectomy performed, and lumbar spinal cord excised and immediately immersed in ice-cold aerated (95% O₂, 5% CO₂) *stabilization buffer* (**SB**; containing, in mM: 139 sucrose, 32.5 NaCl, 2.5 KCl, 10 MgSO₄, 12 Glucose, 24 NaHCO₃, 1 NaH₂PO₄ and 0.5 CaCl₂). After removing the dura, the lumbar enlargement was embedded in 2.5% agar, and transverse slices (500 μ m) cut on a vibratome (Leica VT 1200, Nussloch, Germany). The slices were returned to the aerated SB for 25 minutes (at 4°C) and then brought to room temperature for another 20 minutes prior to beginning the experiment. For stains on embryonic mouse spinal cord, pregnant mice were deeply anesthetized with Isoflurane, decapitated, and spinal cords rapidly removed from E13–14 embryos, placed in ice-cold aerated **SB** and sliced on a tissue chopper (400 μ m); slices were then maintained in ice-cold aerated SB for 25 more min and brought to RT for 20 min, prior to Co²⁺ loading as described for adult slices.

Spinal cord organotypic slice cultures—Sprague Dawley rat pups (P8) were anesthetized with Isoflurane, decapitated, and the spinal cords rapidly removed. Transverse sections (400 μ m) were cut from the lumbar cord with a tissue chopper under sterile conditions, and placed on the surface of Millipore-CM porous (0.4 μ m) membrane inserts (Millipore Corporation, Billerica, MA) suspended in 35mm culture wells (BECTON Dickinson, NJ) containing 1 ml of *incubation medium* (**IM**; consisting of 50% minimal essential medium (MEM), 25% Hank's balanced salt solution (HBSS) and 25% heat inactivated horse serum, modified by supplementation with: 25 mM HEPES; 3 mM glutamine; 1.14mM MgSO₄·7 H₂O; 4mM NaHCO₃; 0.57 mM ascorbic acid, 2 μ g/ml insulin, 6.4 mg/ml D-glucose, pH 7.2–7.3) (Richichi et al., 2008). The medium was changed one day after plating and subsequent media exchanges were carried out twice per week.

After 10 days *in vitro*, some cultures were subjected to the Co²⁺ labeling procedure (as described below) to assess levels of Ca-perm AMPAr. Other slice cultures were subjected to neurotoxicity paradigms, via exposures to either IM alone or with the addition of drugs (30 μ M PDC, 6 nM TNF- α , PDC+TNF- α , PDC+TNF- α + 10 μ M NBQX, or PDC+TNF- α + 150 μ M NASPM). After 48 hours, the slices were fixed with 4% PFA, and processed for histological labeling.

Kainate stimulated Co²⁺ uptake labeling

Acute spinal cord slices were transferred to *uptake buffer* (**UB**; containing, in mM: 139 Sucrose, 57.5 NaCl, 5 KCl, 2 MgCl₂, 1 CaCl₂, 10 HEPES, 12 Glucose), with the addition of H89 dihydrochloride (a PKA antagonist, 10 μ M, Calbiochem, La Jolla, CA), wortmannin (a

non-selective PI3K antagonist, 100 μ M, Tocris Bioscience, Ellisville MI), or DMSO (2 μ l/ml). After a 10 minute pre-incubation, TNF- α (20 nM, Sigma Aldrich, St. Louis, MO) was added to treatment (non-control) groups for 10 more minutes, a separate DMSO pretreatment plus TNF- α vehicle group was included as a control.

Pretreatment/treatment paradigms were all followed by addition of a small volume of concentrated Co^{2+} and kainate (to final conc. 2.5 mM Co^{2+} ; 100 μ M kainate) for 20 more minutes (total TNF- α exposure duration, 30 min). For Co^{2+} loading of slice cultures, cultures were washed into serum free buffer in the presence or absence of 20 nM TNF- α , and after 20 min, washed, and then exposed to 2.5 mM Co^{2+} and 100 μ M kainate in UB, as above. In both paradigms, loading was terminated by a wash with UB containing 2mM Ca-EDTA to remove extracellular Co^{2+} , followed by treatment with $(\text{NH}_4)_2\text{S}$ (0.067%, 5 min) to precipitate intracellular Co^{2+} . Slices (both acute and slice cultures) were fixed overnight (with 4% PFA). Acute slices were next cryoprotected (with 30% sucrose $\times$ 24h), OCT embedding solution and frozen cut to 30 μ m. Intact slice cultures and sub-sectioned acute slices were then subjected to AgNO_3 enhancement of the Co^{2+} stain, carried out in the dark for 60 minutes as described (Yin et al., 1999), before washing again to terminate the exposure.

Quantification of MN Co^{2+} labeling intensity—Prior studies have indicated that the absolute level of Co^{2+} labeling intensity depends critically upon experimental conditions (temperature, Co^{2+} and kainate concentrations and durations of exposure, and development times), and that, within an experiment, relative differences in labeling between cells and across regions of cells correlates with relative numbers of Ca-perm AMPAr present (Ogoshi and Weiss, 2003; Yin et al., 1999). Therefore, in order to derive comparative information about Ca-perm AMPAr levels across treatment conditions, in each experiment (comprising studies from spinal cord sections of one adult rat), a full set of control and treatment conditions were carried out simultaneously using identical exposure times and reagents. Furthermore, in line with our previous experience (Yin et al., 1994; Yin et al., 1999), since staining can saturate, Co^{2+} loading and development conditions need to be calibrated so as to achieve a mid range of labeling intensity of most positive neurons in the control condition, such that there is considerable dynamic range to resolve increases or decreases between treatment conditions. In addition, since tissue damage in cut surfaces of the acute slices results in some nonspecific Co^{2+} uptake, and staining in deeper slices depends upon adequate tissue penetration of the reaction mix, top and bottom sections from each piece were discarded, as well as a small number of slices from the center that are very faintly stained; slices between ~50–150 μ m from either side were kept for measurement.

In six experiments, Co^{2+} labeling intensity of MNs was assessed using Image J software (Public Domain software from the NIH). MNs were identified on identically photographed sets of 400x micrographs from each experiment and all of their somata were outlined to select regions for measurement. The 8 bit intensity of the image was inverted (new value = 256 minus the old value) such that the near white background gets assigned lowest numerical intensity values, and the darkest stained regions received the highest numerical values, and the labeling intensity of each MN was acquired. Values in Fig. 3 represent compilations of all experiments (all 6 included TNF- α and control conditions; H89 and wortmannin were each included in 3), with 5–10 MNs from each of 8–10 thin (30 μ m) slices derived from 5–6 thick (500 μ m) spinal cord sections for each condition in each experiment ($N > 600$ MNs examined for each condition). TNF- α concentration dependence studies (Fig. 2) show a representative dose response curve, incorporating average values from > 20 thin slices (comprising > 100 MNs) for each condition.

Immunohistochemical staining of slice cultures

Immunohistochemical staining was carried out on floating slices. Slices were blocked (10% HS, 1h), and exposed to primary monoclonal anti non-phosphorylated neurofilament antibody (SMI-32, Sternberger Monoclonals, Berkeley, CA), in 10% HS, 0.3% Triton-X 100 (IP, 1:6000; IF, 1:1000; 4°C, 72h). Labeling was visualized by routine immunoperoxidase techniques (using ABC avidin biotin horseradish peroxidase complex solution, Vector laboratories, Burlingame, CA) or by fluorescence using secondary antibodies linked to a fluorophore (DyLightTM594, Jackson immunoResearch, West Grove, PA). For Co^{2+} /SMI-32 double labeling, Co^{2+} loading was carried out as described, followed by SMI-32 immunostaining, photographing, development of the Co^{2+} stain and re-photographing the field.

Quantification of histopathological changes

Surviving SMI-32 labeled MNs were counted in ventral horn of lumbar spinal slices cultures from each condition, and survival is presented as the percentage of numbers present in wild type slices exposed to media only. Data represent counts from 3–11 independent experiments (with 5–15 slices counted for each condition in each experiment). MNs with pyknotic nuclei, or with a markedly atrophic soma or fragmented proximal dendrites were not counted as alive.

Chemicals and Reagents

TNF- α was purchased from Sigma Aldrich (St. Louis, MO), H89 dihydrochloride was obtained from Calbiochem (La Jolla, CA), wortmannin and PDC were obtained from Tocris Bioscience (Ellisville MI), SMI-32 antibody is from Sternberger Monoclonals (Berkeley, CA), and NBQX was kindly provided by Novo Nordisk (Malov, Denmark). Culture media including horse serum (HS), MEM and HBSS are from Invitrogen (Grand Island, NY). All other chemicals and reagents were obtained from common commercial sources.

Results

Ca-perm AMPAr are present in spinal MNs from developing and adult rats

As discussed above, numerous studies have used techniques including kainate stimulated Co^{2+} uptake labeling (“ Co^{2+} labeling”) or electrophysiological recording to demonstrate the presence of Ca-perm AMPAr on embryonic and neonatal MNs. Although it has been widely assumed that adult MNs are likely to possess these receptors as well, the evidence to date for their presence in adults is largely indirect, based upon measurements of relative AMPA subunit mRNA or protein levels (Shaw and Ince, 1997; Williams et al., 1997), or on attenuation of MN injury in animal models by Ca-perm AMPAr blockers or by targeted increases in GluA2 subunit expression in MNs (Corona and Tapia, 2007; Darman et al., 2004; Tateno et al., 2004; Yin et al., 2007).

Therefore, in initial experiments, we used Co^{2+} labeling techniques to assess the presence of functional Ca-perm AMPAr on adult MNs. This technique is based on the ability of Co^{2+} ions to permeate Ca-perm AMPAr gated channels, but not to cross the plasma membrane through the other major routes of excitotoxic Ca^{2+} influx: voltage gated Ca^{2+} channels or NMDA receptors. Thus, upon kainate exposure (to activate the Ca-perm AMPAr) in the presence of Co^{2+} , Co^{2+} accumulates in neurons possessing Ca-perm AMPAr, where it can be precipitated and visualized by silver enhancement (Pruss et al., 1991). The specificity of the stain has been validated in many studies by ourselves and others, and has been used not only to identify populations of neurons possessing substantial numbers of Ca-perm AMPAr, but also to provide information as to the regional distribution of these receptors on the cell

surface (Launey et al., 1998; Ogoshi and Weiss, 2003; Toomim and Millington, 1998; Yin et al., 1999).

Spinal cords were rapidly removed from 55–65 day old rats, and acute lumbar enlargement slices prepared and subjected to Co^{2+} labeling as described (see Materials and Methods). Distinct Co^{2+} labeling under basal conditions was observed in the majority of ventral horn MNs as well as in some dorsal horn neurons (particularly in the superficial dorsal horn, laminae I–III). Staining was observed in both the somata and dendrites of most MNs (Fig. 1A, B). This prominent dendritic Co^{2+} labeling has been noted previously by ourselves and others in immature MNs (Carriedo et al., 1996; Launey et al., 1998), and is consistent with immunocytochemical studies suggesting that AMPA receptors of different subunit compositions are differentially distributed on the MN membrane, with the majority of GluA2 lacking Ca-perm AMPAR present at synapses in dendrites (Vandenberghe et al., 2001). Indicating the specificity of the stain, it was almost completely eliminated by addition of the non-NMDA receptor antagonist 2,3-Dihydroxy-6-nitro-7-sulfamoylbenzo (F) quinoxaline (NBQX; 10 μM) during the Co^{2+} loading (Fig. 1C). In prior control studies, we have also found Co^{2+} labeling of MNs to be blocked by the Ca-perm AMPAR specific antagonist, 1-naphthyl acetylspermine, NASPM (data not shown). By way of comparison, we also show Co^{2+} labeling from an embryonic (E14) mouse spinal cord (Fig 1D); note the clustered immature Co^{2+} labeled MNs, that have not yet finished migrating to their adult positions. To help verify the MN identity of these Co^{2+} labeled neurons, double stains were carried out showing most of the Co^{2+} labeled ventral horn neurons to be immunoreactive for the SMI-32 antibody to non-phosphorylated neurofilaments, which provides excellent staining of MN morphology *in vivo* and in culture (Carriedo et al., 1996) (Fig 1E, adult; and F, embryonic).

TNF- α induces a rapid increase in functional Ca-perm AMPAR numbers on adult MNs

Subsequent experiments made use of Co^{2+} labeling techniques to examine the ability of TNF- α to induce a rapid insertion of Ca-perm AMPAR from the cytosol into the plasma membrane of adult MNs. Although this technique has most often been used to simply discriminate the presence or absence of Ca-perm AMPAR in a neuronal population, it is clear that labeling intensity can be graded over a wide range from barely perceptible to saturating depending upon factors including the extracellular Co^{2+} concentration, the agonist concentration, or the duration of the Co^{2+} loading exposure, reflecting the extent of intracellular Co^{2+} ion accumulation through Ca-perm AMPAR. Indeed, Co^{2+} labeling intensity has been previously used to assess changes in Ca-perm AMPAR numbers after TNF- α exposure in hippocampal pyramidal neurons (Ogoshi et al., 2005), as well as in individual cerebellar purkinje neurons in slice (Bliss et al., 2011). Of note, however, since labeling intensity is both highly sensitive to loading and development conditions and is saturable, the utility of the technique for making reliable comparisons between Ca-perm AMPAR levels requires care in the establishment of baseline Co^{2+} loading parameters, and comparisons across all conditions must be made in each experiment, as discussed (see Materials and Methods).

In an initial study, lumbar spinal cord slices were obtained from 55–65 day old rats as above and subjected to Co^{2+} labeling in the absence of drug treatment, or after incubation with 6, 12 or 20 nM TNF- α for 30 min. This dose response data was obtained both to ascertain the range of concentrations over which TNF- α could induce rapid increases in Ca-perm AMPAR numbers and to provide information as to the sensitivity of the Co^{2+} labeling technique to resolve and discriminate levels of new Ca-perm AMPAR incorporation.

Addition of 6 nM TNF- α caused a modest but distinct increase in the intensity of Co^{2+} labeling in MNs, in comparison to the control (0 TNF- α condition), and 12 nM TNF- α

resulted in a further distinct increase in labeling intensity. However, with 20 nM TNF- α exposures, labeling looked largely the same as with 12 nM (Fig. 2). Thus, it appears that with 30 min exposures, the number of membrane Ca-perm AMPAr increases over the low nM range of TNF- α exposures, with 6 nM yielding a modest increase, and 12 nM likely yielding near maximal increases in Ca-perm AMPAr numbers. Of note, the effects of TNF- α were not strictly selective for MNs, as some dorsal horn neurons, especially in superficial laminae I–III, also showed an increase in labeling (data not shown).

We next set out to examine signaling pathways involved in the rapid TNF- α induced membrane insertion of Ca-perm AMPAr in MNs. Prior studies in forebrain neurons have found TNF- α induced membrane insertion of new AMPA receptors to be phosphatidylinositol 3 kinase (PI3K) dependent (Leonoudakis et al., 2008; Stellwagen et al., 2005) and have reported that phosphorylation of the GluA1 AMPA subunit at serine 845 by protein kinase A promotes receptor insertion into the plasma membrane and decreases endocytosis (Man et al., 2007). Lumbar spinal cord slices were subjected to Co²⁺ labeling as above, in the absence of drug treatment, after incubation with 20 nM TNF- α alone (30 min), or in the additional presence of the phosphatidylinositol 3 kinase antagonist, wortmannin (100 μ M), or the protein kinase A antagonist, H89 (10 μ M). After development of the Co²⁺ stain, staining intensity was quantified as described (see Materials and Methods). In 6 experiments (in each of which >200 MNs were evaluated for each condition; see Materials and Methods), TNF- α caused a distinct, significant increase in the intensity of Co²⁺ labeling, indicative of the membrane insertion of new Ca-perm AMPAr. Furthermore, the increased Co²⁺ labeling was largely prevented by the presence of either the non-selective PI3K antagonist, wortmannin or the PKA antagonist, H89 (each of which was used in 3 of the 6 experiments), indicating that both PI3K and PKA pathways are involved in the signaling leading to the receptor trafficking from the cytosol into the membrane (Fig. 3).

TNF- α potentiates slow excitotoxic MN injury in organotypic slice cultures

To begin to assess the possible contributions of TNF- α to chronic MN degeneration, as may be applicable to ALS, we made use of rat lumbar spinal organotypic slice cultures. These cultures have several distinct advantages for studies of neurodegenerative mechanisms. First, unlike dissociated cultures, slice cultures can survive for weeks, largely preserving the native cellular architecture. This is essential for modeling disease associated MN loss, as it is increasingly clear that the pathogenesis of ALS and other diseases of the spinal cord 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.

Slices were prepared from 8 day old rat pups, and plated on membrane inserts suspended in media as described. After an additional 10 days *in vitro*, slice cultures were either subjected to Co²⁺ labeling (with or without 20 min pre-exposure to 20 nM TNF- α), or chronic pharmacological exposures were initiated. Co²⁺ labeling was carried out largely as described for acute slices (see Materials and Methods). Distinct labeling was consistently observed in untreated ventral horn MNs in these cultures, again with pronounced labeling often seen in dendrites (Fig. 4A, C). This was consistent with prior observation in slice cultures from embryonic spinal cord (Launey et al., 1998). However, brief exposures to 20 nM TNF- α caused a dramatic increase in the intensity of the Co²⁺ labeling, similar to that seen in the acute slices from the adult animals (Fig. 4B, D). This rapid effect of TNF- α indicates that MNs in this preparation are capable of strongly upregulating their surface Ca-perm AMPAr in response to appropriate stimuli.

For the chronic neurotoxicity experiments, the cultures were exposed for 48h to buffer, to TNF- α (6 nM) or to the glutamate uptake blocker, *trans*-pyrrolidine-2,4-dicarboxylic acid

(PDC; 30 μ M) alone, or to TNF- α +PDC, or TNF- α +PDC along with the non-NMDA receptor antagonist, NBQX (10 μ M) or with the Ca-perm AMPAR selective blocker, NASPM (150 μ M). At the end of the 48h exposure, the slices were immunostained with the SMI-32 antibody to non-phosphorylated neurofilaments, and MN survival was assessed by direct counting of intact ventral horn MNs. We found that whereas the exposures to either TNF- α or to PDC alone caused little MN damage, when combined, substantial MN degeneration occurred. Furthermore, this damage was substantially decreased by either NBQX or NASPM. (Fig. 5, inserts show blow ups of boxed regions of ventral horns, top, and dorsal horns, bottom). Note that dorsal horn neurons are relatively preserved in all conditions (in over 80% of > 50 slices exposed to PDC+TNF- α degenerative changes were distinctly greater in the ventral horn MNs compared to dorsal horn neurons; in the remaining slices there was relatively little MN damage).

Interestingly, Co²⁺ labeling of these slice cultures after the 48h 6 nM TNF- α exposures did not show the dramatic increases in labeling seen after acute 12 or 20 nM TNF- α exposures (data not shown). This may be consistent with our observations that 6 nM TNF- α induced relatively modest increases on Co²⁺ labeling with acute exposures, and with observations in hippocampal neurons that TNF- α can also cause membrane insertion of new GluA2 containing Ca²⁺ and Co²⁺ impermeable receptors (Ogoshi et al., 2005), which appear to be inserted with a slower time course than the GluA2 lacking receptors (Leonoudakis et al., 2008). However, it remains likely that prolonged TNF- α exposures result in new Ca-perm AMPAR in some cellular domains and further studies will be needed to more precisely determine ways in which lower and more prolonged TNF- α exposures alter distribution of Ca-perm AMPAR that may predispose MNs to excitotoxic injury.

Discussion

Summary of principal findings

In the present study, we use a well-established histochemical technique to directly demonstrate, for the first time, the presence of functional Ca-perm AMPAR on adult MNs, and find that acute TNF- α exposure induces a rapid increase in the numbers of these receptors in the neuronal membrane, via a signal transduction pathway that includes both PI3K and PKA. We further find that MNs in organotypic spinal cord slice cultures possess these Ca-perm AMPAR under basal conditions (after 10 days *in vitro*), and that, as in adult slices, acute TNF- α exposures caused an abrupt, dose dependent increase in Ca-perm AMPAR numbers on their plasma membranes. Finally, using these slice cultures, we find that whereas prolonged low level glutamate uptake blockade or exposure to low dose TNF- α alone caused little injury, combined exposure induced pronounced and relatively selective MN degeneration, via a Ca-perm AMPAR dependent mechanism.

Ca-perm AMPAR and excitotoxic neurodegeneration

Excitotoxicity, or neuronal damage caused by exposure to excessive levels of extracellular glutamate, is strongly implicated in the neurodegeneration occurring in conditions of ischemia, prolonged seizures and trauma, in which glutamate rapidly accumulates in the extracellular space. In these conditions, most attention has been placed upon the contributory roles of highly Ca²⁺ permeable NMDA type glutamate receptors in the induction of injury, resulting from rapid neuronal Ca²⁺ influx. However, there is also considerable evidence for contributions of AMPA type glutamate receptors not only in these acute conditions, but in the slowly evolving neurodegeneration occurring in degenerative conditions like ALS or Alzheimer's. Whereas most AMPA receptors are Ca²⁺ impermeable, it appears that Ca-perm AMPAR, which are only present on subpopulations of neurons, may be of particular importance to selective excitotoxic neurodegeneration (Kwak and Weiss,

2006; Weiss and Sensi, 2000). There are several likely reasons for the important contributions of these channels. First, unlike NMDA channels, which are blocked by Mg^{2+} ions in a voltage dependent fashion and therefore only pass significant current when the postsynaptic neuron is depolarized, Ca-perm AMPAr permit passage of Ca^{2+} whenever the receptor is activated by glutamate.

In addition, besides providing a fixed route for glutamate activated Ca^{2+} influx into neurons, a large number of studies have shown that numbers of Ca-perm AMPAr are highly subject to regulation by physiological events or environmental factors. Acute conditions like ischemia and trauma, in which excitotoxicity prominently contributes, can lead to increases in Ca-perm AMPAr in brain or spinal neurons via mechanisms including long term downregulation of GluA2 expression (Gorter et al., 1997; Grossman et al., 1999) and internalization of Ca^{2+} impermeable AMPA receptors with membrane insertion of new Ca-perm AMPAr (Bell et al., 2009; Liu et al., 2006). Thus, it is likely that disease associated increases in their numbers would subject affected neurons to a new metabolic burden that may promote injury in neurons that are already stressed by disease.

Ca-perm AMPAr in disease associated degeneration of spinal MNs

Despite the clear demonstration of Ca-perm AMPAr on embryonic and immature spinal MNs under basal conditions (Carriedo et al., 1995; Carriedo et al., 1996; Van Den Bosch et al., 2000; Vandenberghe et al., 2000), most studies have found the GluA2 AMPA subunit, which blocks Ca^{2+} permeability of AMPA receptors, to be present in MNs as well. Thus, Ca^{2+} permeable and impermeable AMPA channels co-exist on most MNs, and it appears that GluA2 lacking (Ca^{2+} permeable) AMPA receptors are disproportionately found in the dendrites (Launey et al., 1998; Van Den Bosch et al., 2000; Vandenberghe et al., 2001), much as has been described for hippocampal pyramidal neurons (Ikonomovic et al., 1995; Lerma et al., 1994; Ogoshi and Weiss, 2003; Toomim and Millington, 1998; Yin et al., 1999).

Interestingly, no prior studies have directly examined the presence of Ca-perm AMPAr present on adult MNs. However, a number of studies of spinal cord disease models provide indirect evidence for their presence on adult MNs. Specifically, observations that modulation of Ca-perm AMPAr numbers in MNs bidirectionally modulates disease progression in SOD1 mutant mouse models of ALS strongly suggests a contributory role of these receptors, at least in that model (Kuner et al., 2005; Tateno et al., 2004; Van Damme et al., 2005). In addition, Ca-perm AMPAr blockers protect MNs from injury after intrathecal AMPA infusions (Corona and Tapia, 2007), slow MN degeneration SOD1 mutant rats (Yin et al., 2007), and attenuate MN injury in spinal cord organotypic slice cultures from SOD1 mutant rats subjected to partial glutamate uptake inhibition (Yin and Weiss, 2012). Ca-perm AMPAr likely contribute to MN loss in other conditions as well; spinal cord contusion (Grossman et al., 1999) and ventral root evulsion (Nagano et al., 2003) were reported to induce loss of GluA2 in spinal MNs, and a Ca-perm AMPAr blocker attenuated MN loss caused by infection with a neurotrophic virus (Darman et al., 2004). Interestingly, despite the presence of Ca-perm AMPAr in many dorsal horn neurons, we found MNs to be preferentially injured by the prolonged PDC+TNF- α exposures, consistent with findings of preferential MN degeneration in diseases including ALS. Factors downstream from Ca^{2+} entry through Ca-perm AMPAr that may underlie the high susceptibility of MNs to excitotoxic injury and propensity to degenerate in disease include poor cytosolic Ca^{2+} buffering capacity (Alexianu et al., 1994; Elliott and Snider, 1995; Lips and Keller, 1998; Vanselow and Keller, 2000), with the consequence that the Ca^{2+} loads are rapidly taken up by mitochondria, resulting in strong mitochondrial ROS generation (Carriedo et al., 2000).

Neuroinflammation and TNF- α in disease

Neuroinflammation plays an integral role in many diseases of the nervous system, from immune mediated diseases of the nervous system like multiple sclerosis, to acute conditions like ischemia or trauma, infectious diseases, and neurodegenerative diseases. The neuroinflammatory response is largely mediated by glial cells, both microglia and astrocytes, which, when “activated”, change their function and can release a host of cytokines and other neuroactive factors. In the spinal cord, neuroinflammation has been strongly linked to MN degeneration in conditions from ALS (Philips and Robberecht, 2011) to viral infection (Darman et al., 2004; Zachary et al., 1997).

TNF- α can be produced both in astrocytes and microglia, and has complex effects which may be either injurious or protective; for this reason, the net effect of TNF- α on the course of ALS and other neurodegenerative disease is presently uncertain (Ghezzi and Mennini, 2001; Hensley et al., 2006; Mhatre et al., 2004; Pickering et al., 2005; Tweedie et al., 2007). Interestingly, presently described membrane insertion of new AMPA receptors in response to TNF- α , in addition to having deleterious effects in disease, may ordinarily be of physiological importance, for instance, in homeostatic synaptic plasticity, in which glial TNF- α and PI3K appear to play roles in signaling the membrane incorporation of new AMPA receptors in underactive synapses (Hou et al., 2008; Stellwagen and Malenka, 2006). Another mechanism through which TNF- α may promote excitotoxic injury is via interfering with astrocyte glutamate uptake (Carmen et al., 2009; Fine et al., 1996; Zou and Crews, 2005), and this effect may also play a role in excitotoxic MN degeneration caused by prolonged TNF- α exposures (Tolosa et al., 2011).

Conclusions

It is clear that the survival of MNs depends critically upon interrelationships with neighboring cells, and neuroinflammation, with astrocyte dysfunction, likely contributes to MN damage in a number of conditions including ALS. TNF- α is an important cytokine that accumulates in diseased spinal cord, and has complex effects that could well modulate MN survival. However, as discussed above, it has differing effects in different *in vitro* and animal models, and its net effects on MN survival in ALS or other conditions remains uncertain.

In the present studies, we provide the first direct demonstration that substantial numbers of Ca-perm AMPAR are present on adult MNs under basal conditions, and that their numbers can be rapidly increased by exposure to TNF- α . We further make use of slice cultures, in which MNs are incorporated within their native glial matrix, to model effects of TNF- α on slow MN degeneration. We find that low levels of TNF- α markedly potentiated the MN injury caused by normally subtoxic levels of glutamate uptake blockade. Thus, although the role of TNF- α in ALS and other conditions is not firmly established, present findings are consistent with the idea that TNF- α promotes slow MN injury, likely in part via upregulation of Ca-perm AMPAR.

As MN survival in disease is critically dependent upon complex environmental factors and interactions with neighboring cells, it may be unlikely that we will find single interventions that halt the pathogenic process. However, the progressive understanding of factors which may either act to maintain or disrupt the homeostasis permitting MN survival may suggest sets of interventions acting at distinct and synergistic mechanisms, which, in combination, will yield significant efficacy.

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Abbreviations

ALS	amyotrophic lateral sclerosis
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
Ca-perm AMPAR	Ca ²⁺ permeable AMPA receptors
MN	motor neuron
NASPM	1-naphthyl acetylspermine
NBQX	2,3-Dihydroxy-6-nitro-7-sulfamoylbenzo (F) quinoxaline
PI3K	phosphatidylinositol 3 kinase
PKA	protein kinase A
SOD1	superoxide dismutase type 1
PDC	<i>trans</i> -pyrrolidine-2,4-dicarboxylic acid
TNF-α	tumor necrosis factor-alpha

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Highlights

- First direct demonstration of functional Ca^{2+} permeable AMPA receptors in adult MNs
- TNF- α rapidly increases numbers of these receptors in the plasma membrane
- This TNF- α effect occurs via a PI3K and PKA dependent mechanism
- TNF- α also increases MN Ca^{2+} permeable AMPA receptors in slice cultures
- Low level TNF- α exposure enhances slow excitotoxic MN injury in slice cultures

Figure 1.

Kainate stimulated Co^{2+} uptake labeling confirms the presence of Ca-perm AMPAR in adult MNs. Acute lumbar spinal cord sections from 55–65 day old rats were subjected to kainate stimulated Co^{2+} uptake labeling as described. Note the distinct Co^{2+} accumulation in ventral horn MNs (A, low magnification; B, detail of ventral horn). Indicating the specificity of the stain, it was substantially prevented by the addition of the non-NMDA (AMPA and kainate) receptor antagonist, NBQX (10 μM) during the kainate + Co^{2+} exposure (C). For comparison, studies of embryonic animals (in the illustrated case, E14 mice) show Co^{2+} labeling in immature ventral horn MNs (D). Note that the immature Co^{2+} labeled MNs are clustered, and are in the process of migrating to their adult positions. Inserts (C, D) show high power view of ventral horn regions marked by squares. Double labeling indicates that many of the putative ventral horn MNs that are identified by kainate stimulated Co^{2+} uptake labeling are also immunoreactive for the SMI-32 antibody in both adult (E; left, SMI-32 IF; right, Co^{2+} labeling) and embryonic animals (F, left, SMI-32 IP; right, Co^{2+} labeling). In F, note the absence of SMI-32 labeling, but the accumulation of Co^{2+} labeling reaction product in the nuclei of identified neurons. Bar = 500 μm (A, C); 100 μm (B, E, insert C); 200 μm (D); 50 μm (F, insert D).

Figure 2.

TNF- α induces rapid insertion of new Ca-perm AMPAr in membranes of adult MNs in a concentration dependent fashion.

A. Representative images: Acute lumbar spinal cord sections from 55–65 day old rats were subjected to kainate stimulated Co²⁺ uptake labeling, either alone, after incubation with 6, 12 or 20 nM TNF- α , as described. Photomicrographs show ventral horn regions of Co²⁺ labeled sections from one experiment. Bar = 100 μ m.

B. Representative dose response curve. After development of the Co²⁺ stain, staining intensity was quantified as described (see Materials and Methods). Data represent compilation of mean intensity values from >20 slices (with > 100 MNs measured) each condition. * indicates difference from 0 TNF- α ; # indicates difference from 6 nM TNF- α ($p < 0.01$) by Kruskal-Wallis one way ANOVA on ranks with Student-Newman-Keuls post hoc test.

Figure 3.

TNF- α induces rapid insertion of new Ca-perm AMPAr receptors in membranes of adult MNs via a PI3K and PKA dependent mechanism.

A. Representative images: Acute lumbar spinal cord sections from 55–65 day old rats were subjected to kainate stimulated Co²⁺ uptake labeling, either alone, after incubation with 20 nM TNF- α , or in tissue that had been pretreated with the PI3K antagonist, wortmannin, or the PKA antagonist, H89, as described. Photomicrographs show ventral horn regions of Co²⁺ labeled sections from one experiment. Bar = 100 μ m.

B. Quantitative assessment. After development of the Co²⁺ stain, staining intensity was quantified as described (see Materials and Methods). Data represent compilation of 6 experiments comprising TNF- α and control conditions, with H89 and wortmannin each included in 3 (with >600 MNs counted each condition). * indicates difference from the control, TNF- α +H89 and TNF- α +wortmannin ($p < 0.02$) by Kruskal-Wallis one way ANOVA on ranks with Student-Newman-Keuls post hoc test.

Figure 4.

Ca-perm AMPAr are present in MNs spinal cord slice cultures and their numbers are rapidly increased by TNF- α exposure. Organotypic spinal cord slice cultures were produced from 8 day old rat pups, and maintained for 10 more days *in vitro* prior to subjecting them to kainate stimulated Co^{2+} uptake labeling either in the absence of TNF- α exposure (A, C) or after 20 min exposure to 20 nM TNF- α (B, D). C and D show high power views of ventral horn regions marked by squares in A and B. Note the distinct Co^{2+} labeling, often evident in dendrites, of untreated slice cultures, and the marked intensification of the labeling after TNF- α exposures. Photomicrographs are representative of 4 experiments. Bar =400 μm (A, B), or 50 μm (C, D).

Figure 5.

TNF- α markedly potentiates slow MN degeneration caused by low level glutamate transport blockade in slice cultures via an AMPA receptor dependent mechanism. Organotypic spinal cord slice cultures were produced from 8 day old rat pups, and kept *in vitro* for 10 more days prior to exposure for 48h to buffer alone, with PDC (30 μ M) or TNF- α (6 nM) alone, or to PDC+TNF- α alone, or with the non-NMDA receptor antagonist, NBQX (10 μ M) or the Ca-perm AMPAR selective blocker, NASPM. At the end of the 48h exposure, the slices were immunostained with SMI-32 to examine MN morphology.

A. Representative photomicrographs. Low power images show spinal cord hemi-sections; details show blow ups of boxed regions (ventral horn, top; dorsal horn, bottom). Note the paucity of MN injury caused by exposure to either PDC or TNF- α alone, the marked potentiation of injury by combined exposure to PDC+TNF- α , and the substantial attenuation

of this injury by NBQX. Further note the paucity of damage to dorsal horn neurons in the PDC+TNF- α condition despite extensive damage to ventral horn MNs. Bar = 50 μ m.

B. Quantitative assessment. MN survival was assessed by direct counting of intact ventral horn MNs, as described (values are compiled from 3–11 independent experiments (with 5–15 slices counted for each condition in each experiment). * indicates difference from all other conditions ($p < 0.01$) by Kruskal-Wallis one way ANOVA on ranks with Student-Newman-Keuls post hoc test.

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