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Astroglia-mediated effects of uric acid to protect spinal cord neurons from glutamate toxicity

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Abstract:

Uric acid has been demonstrated to reduce damage to neurons elicited by oxidative stress. However, our studies utilizing cultures derived from embryonic rat spinal cord indicate that an astroglia-mediated mechanism is involved in the effects of uric acid to protect neurons from glutamate toxicity. The damage elicted by glutamate to neurons in a mixed culture of spinal cord cells can be reversed by uric acid. Furthermore, addition of uric acid after the termination of glutamate exposure suggests that uric acid plays an active role in mediating neuroprotection rather than purely binding peroxynitrite, as previously thought. Importantly, in pure neuron cultures from the same tissue, uric acid does not protect against glutamate toxicity. Addition of astroglia to the pure neuron cultures restores the ability of uric acid to protect the neurons from glutamate-induced toxicity. Our results also suggest that glia provide EAAT-1 and EAAT-2 glutamate transporters to protect neurons from glutamate and that functional EAATs may be necessary to mediate the effects of UA. Taken together, our data strongly suggest that astroglia in mixed cultures are essential for mediating the effects of uric acid, revealing a novel mechanism by which UA, a naturally produced substance in the body, may act to protect neurons from damage during insults such as spinal cord injury.

Introduction

Neurons are extremely vulnerable to injury during pathological conditions, including stroke and spinal cord damage. The process of neuronal death resulting from spinal cord injury (SCI) involves glutamate receptor overstimulation as a result of tissue damage, ischemic cell death, and synaptic and non-synaptic transport of glutamate (Liu et al., 1991; Liu et al., 1999; McAdoo et al., 1999). Impaired mitochondrial function and excess calcium influx follows (Choi, 1996; Yu et al., 1998) , resulting in neuronal apoptosis and necrosis (McAdoo et al., 1999). N-methyl-D-asparate (NMDA) and alpha-hydroxy-5-methyl-4-isoazole-propionic acid (AMPA) receptors have been shown to play central roles in the cellular damage caused by glutamate. Although antagonists to these glutamate receptors have shown promising improvements for histological damage and functional deficits in experimental animal models of SCI (Faden et al., 1988; Gomez-Pinilla et al., 1989; Faden et al., 1990; Liu et al., 1997; Wrathall et al., 1997), these compounds fail to yield successful clinical results (Doppenberg et al., 1997). Therefore, it is important to identify other neuroprotective and regenerative agents that fight glutamate toxicity after SCI.

Uric Acid (UA) is an anti-toxicity agent that is naturally prevalent in the body (Becker, 1993; Ryan et al., 1997). Recent studies have demonstrated possible roles played by UA in protecting CNS neurons from excitotoxic and metabolic insults. For example, UA has been demonstrated to protect hippocampal neurons from glutamate toxicity (Yu et al., 1998) and cortical neurons from peroxynitrite-elicted damage (Trackey et al., 2001). Furthermore, Hooper and colleagues found that treatment with UA in a mouse model of multiple sclerosis improves recovery and slows disease progression (Hooper et al., 1998). Although UA has been implicated in reducing oxidative damage to spinal cord neurons (Scott et al., 2005), it is possible that this is not the sole mechanism by which UA acts to block glutamate toxicity in these neurons. Our current study utilizes embryonic rat spinal cord cultures to demonstrate that uric acid can act to protect neurons from glutamate-induced toxicity after termination of glutamate

exposure and that astroglial cells play a central role in mediating the actions of UA.

Our initial experiments demonstrate that UA blocks glutamate-induced neurotoxicity in mixed cultures. Most importantly, even when UA is added after glutamate treatment, it can still act to protect neurons from cell death. However, addition of UA does not reduce the neuronal cell loss after glutamate treatment in pure spinal cord neuronal cultures, suggesting that other cell types present in the mixed culture are crucial for protection by UA. Further studies employing pure astroglial cultures and co-culture of astroglia and neurons indicate that GFAP+ and vimentin+ astroglia are essential for UA action. The importance of astroglial cells was further confirmed by re-introducing these cells into pure neuron cultures, and this re-introduction restored the effects of UA to protect neurons. Moreover, our data suggest that excitatory amino acid transporters (EAATs), which are expressed by astroglial cells, are likely to play a role in this protection since blocking glutamate transporters abolishes the effects of UA. Thus, our experiments demonstrate a novel action of UA on the glial population in spinal cord cell culture to indirectly protect neurons from glutamate toxicity.

Methods and Materials

Spinal cord neuron cultures:

Spinal cords were dissected from embryonic day 16 (E16) rat embryos. Meninges were removed and the cords were dissociated with gentle trituration. Cells were plated at a density of 350 neurons/mm^2. The mixed cultures were grown in serum-containing medium (SCM; 89.4% MEM (Minimum Essential Medium; Gibco), 10% horse serum, 0.6% glucose, supplemented with penicillin and streptomycin) for 6 days at 37°C and 5% CO_2 before treatment.

To grow pure spinal neuron cultures, SCM was changed to Neurobasal (NB) medium (Gibco) supplemented with B-27, penicillin, and streptomycin at 24 hours after plating. After an additional 24 hours, cytosine arabinoside (Ara-C, 5

μM) was added to these cultures for 3 days after which the Ara-C containing media was changed to fresh NB media. Cells were treated 24 hours later.

Spinal cord astroglial cultures were derived from postnatal day 1 rats using a modified approach from MaCarthy and de Vellis (McCarthy and deVellis, 1980). Spinal cords were dissected and dissociated with trituration. Cells were plated in tissue culture flasks at density of 1000 cells/mm^2. Cultures were grown in NM-15 medium (84.4% MEM, 15% Fetal Bovine Serum, 0.6% glucose, supplemented with penicillin and streptomycin) for 9 days. Medium was changed every 3 days. The flasks were then shaken at 400 x rpm for 20 minutes before the medium was changed and the floating cells (mostly microglia) were washed away. The flasks were again shaken overnight at 250 x rpm to remove attached oligodendrocytes and remaining microglia Follow ing shaking, floating cells were washed away and medium was changed. Ara-C was added to final concentration of 10 μM to reduce the number of undifferentiated cells. Cultures were grown for 3 additional days before being trypsinized and re-plated at a density of 500 cells/mm^2 in SCM. These cultures only contain GFAP+ and vimentin+ astroglia. Staining for MAP-2 and CNPase were negative.

To examine whether soluble factors from astroglia are involved in actions of UA, astroglial cultures were grown in SCM for 3 days before the medium was changed to NB. After another 3 days, UA was added to some cultures while other cultures were treated with Locke's buffer as vehicle for 24 hours. Condition medium (CM) from all cultures was collected. CM from UA treated group still contains UA, while CM from the control group does not. The different CM was used in experiments to evaluate the possible involvement of UA-elicited soluble factors.

To determine the contribution of direct astroglial cell presence to the neuroprotective effects of UA, astroglial cultures were grown for 5 days in SCM before the cells were trypsinized and plated onto pure spinal cord neuron cultures grown for 5 days *in vitro* (DIV 5). The co-culture was grown in SCM and treated 24 hours later.

Treatments:

Reagents used to treat cells were made into stock solutions. Glutamate and uric acid (UA) were dissolved in Locke's buffer (NaCl, 154 mM; KCl, 5.6 mM; $CaCl_2$, 2.3 mM; $MgCl_2$, 1.0 mM; $NaHCO_3$, 3.6 mM; glucose, 5 mM; Hepes, 5 mM; pH 7.2) and diluted as indicated. To induce peroxynitrite toxicity, Sin-1 (3-morpholinoesydnonimine; Sigma), a peroxynitrite donor, was dissolved in PBS and used to treat cells at the indicated concentrations. L-Threohydroxy aspartate (THA; Sigma), an inhibitor of EAATs, was used to block glutamate transporter function. THA was dissolved in PBS.

When cells were treated with glutamate, the medium was replaced with solutions of glutamate in Locke's buffer, with or without UA, for 1 hour at 37°C. In control cultures, the medium was replaced with Locke's buffer. After 1 hour, glutamate solutions were changed to SCM or NB and UA was added at the same concentration. In all cases, same amount of Locke's buffer was added to control as vehicle. When the cultures were treated with Sin-1, the drug was added directly to the medium with or without UA, and the same amount of PBS and Locke's buffer were added to control as vehicle. In all cases, cells were incubated for another 24 hours at 37°C before being fixed or harvested.

Immunocytochemistry:

Specific primary antibodies were used to identify different type of cells in the culture using immunocytochemistry. Monoclonal anti-MAP-2 (microtubule associated protein 2; BD Pharmingen) was used to identify neurons; monoclonal anti-GFAP (glial fibrillary acidic protein; Chemicon Inc.) was used to identify astrocytes; monoclonal anti-CNPase (2'3'-cyclic-nucleotide 3'-phosphodiesterase; Chemicon Inc.) was used to identify oligodendrocytes; monoclonal anti-vimentin (Chemicon Inc.) was used to identify astrocyte progenitors; and monoclonal anti-CD11 (clone OX42; Serotec Inc.) was used to identify microglia.

Cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature. After washing with PBS, the cultures were incubated with anti-MAP-

2 (1:500), anti-GFAP (1:1000), anti-CNPase (1:500), anti-vimentin (1:1000) or anti-CD11 (1:500) for 2 hours at room temperature. Positive cells were then visualized with Cy2 anti-mouse secondary antibodies.

For double labeling of GFAP and EAAT-1 or vimentin and EAAT-2, fixed cells were incubated with mixed solutions of monoclonal anti-GFAP (1:1000) and guinea pig polyclonal anti-EAAT-1 (Chemicon Inc., 1:1000) or mixed solutions of monoclonal anti-vimentin (1:2000) and guinea pig polyclonal anti-EAAT-2 (Chemicon Inc., 1:1000) at room temperature for 2 hours. After rinsing, cultures were visualized with Cy2 anti-mouse and Cy3 anti-guinea pig secondary antibodies.

Cell count and statistics:

For each well, pictures were taken for 10 random fields at a magnification of 100X. Cell number (as indicated by the appropriate immunostain) in each of the 10 pictures was counted by an experimenter blind to the condition. Cell numbers in control and treated groups were compared with one-way analysis of variance (ANOVA) using the Instat software program (Graph Pad). Appropriate multiple comparisons tests were performed as indicated in figure legends and the text.

Results

UA protects spinal cord neurons from glutamate-induced toxicity.

Glutamate is the major physiological agent that mediates cell death after spinal cord injury. To examine the survival of spinal cord neurons in our *in vitro* model of SCI, we treated mixed cell cultures derived from embryonic 16 (E16) rat spinal cords with a range of concentrations of glutamate. These cells were grown in serum-containing medium (SCM) for 6 days (DIV 6) before being treated with various concentrations of glutamate for 1 hour. The brief exposure to high levels of glutamate mimics its temporary up-regulation after SCI. After glutamate was removed, cells were kept in SCM for another 24 hours before the

cultures were fixed and immunostained. The numbers of surviving neurons were monitored with immunohistochemical labeling of MAP-2, a marker for neurons.

The staining for MAP-2 demonstrated that the surviving neurons bear multiple processes and intact membrane structures (Figure 1 A). As shown in Figure 1 B, glutamate-elicited neuronal cell death increased in a dose-dependent manner. With high concentrations of glutamate (500 μM and 1 mM), approximately 40% of the neurons in these cultures did not survive (Figure 1 B).

To determine whether UA can be used to prevent glutamate toxicity, initial experiments monitored the effects of UA on spinal cord neuron survival during basal conditions. DIV 6 cultures were treated with different concentrations of UA for 24 hours before being fixed and immunostained, and the numbers of neurons were counted. UA had no effect on the survival of spinal cord neurons (Figure 2). Further experiments were designed to reveal if UA could reduce neuronal cell death after glutamate exposure. Cells were treated with 500 μM glutamate with or without various concentrations of UA. UA was applied with glutamate and the same concentration of UA was added after the removal of glutamate. As shown in Figure 3 A, UA blocked glutamate toxicity in a dose-dependent manner. With high concentrations of UA, glutamate-promoted neuronal cell death was abolished (Figure 3 A). Most importantly, treatment of UA solely after the termination of glutamate exposure resulted in similar neuroprotection (Figure 3 B). These results suggest that UA can act to protect neurons after glutamate exposure in culture.

UA cannot protect spinal cord neurons from glutamate toxicity in pure neuron cultures

Spinal cord cells grown in SCM are mixed cultures. To identify the cell types present, DIV 6 cultures were immunostained for MAP-2, GFAP, vimentin, CNPase, and CD11. MAP-2 is a marker for neurons, GFAP and vimentin for astroglia, CNPase for oligodendrocytes, and CD11 for microglia. While MAP-2+ neurons, GFAP+ or vimentin+ astroglia, and CNPase+ oligodendrocytes were

identified (Figure 4 A, C, E, G), staining for CD11 was negative (data not shown), indicating the absence of microglia in these cultures.

The presence of astrocytes and oligodendrocytes in mixed cultures suggests the possibility that the effects of UA may not be mediated directly by neurons, but by glial cells which indirectly confers neuronal protection. To address this question, pure spinal cord neuron cultures were established. Twenty four hours after plating in SCM, cells were changed to NB medium, which optimizes neuronal growth. Ara-C (5 μM) was added to the cultures after another 24 hours to eliminate the glial populations. The medium was changed after 3 days, and cells were treated 24 hours later. This procedure eliminated non-neuronal cell types in the cultures with only neurons surviving. Like the neurons grown in SCM, the pure neuron cultures demonstrated that the neurons had extended processes and have intact membrane structures (Figure 4 B). However, staining for GFAP, vimentin, CNPase (Figure 4 D, F, H), and CD11 was absent (data not shown), suggesting that these cultures are a pure neuronal population.

Dose response experiments demonstrated that these cultures are much more sensitive to glutamate toxicity. Treatment with 10 μM glutamate resulted in very significant neuron loss (more than 80%; Figure 5), and 500 μM glutamate essentially eliminated all neurons (Figure 5), compared to only 40% neuronal loss in the mixed cultures treated with the same concentration of glutamate (500 μM; Figure 1). To examine whether UA can directly reduce glutamate toxicity in these cultures, cells were treated with or without various concentrations of UA either concurrent with or at the termination of exposure to 10 μM glutamate. UA itself had no effect on neuron survival, and in addition, it did not show any protection against glutamate toxicity (Figure 6). High concentrations of UA (up to 200 μM) did not alter the neuron loss elicited by 10 μM glutamate. In contrast, toxicity elicited by the peroxynitrite donor, Sin-1, was significantly reversed by the concurrent presence of UA (Figure 7 A). However, when UA was added after Sin-1, it did not elicit a reversal of toxicity (Figure 7 B). These data suggest that peroxynitrite is probably not the major mediator of

glutamate-induced toxicity since 1) UA can protect against Sin-1 toxicity while having no effect on glutamate-induced toxicity in pure neuronal cultures and 2) UA cannot protect neurons from Sin-1-induced toxicity when added after Sin-1 exposure. In addition, these data demonstrate that UA is not likely to affect neurons directly. Non-neuronal cells, most likely astroglia, may mediate the effects of UA to protect neurons from glutamate treatment.

Astroglia play an important role in mediating the effects of UA

One candiate cell type that may mediate the effects of UA is the astroglial population. Astroglia, including GFAP+ and vimentin+ cells, have been reported to protect neurons from excitotoxic insults in CNS trauma (Faulkner et al., 2004; Diaz et al., 2005). As such, our further studies examined whether astroglia contribute to the effects of UA.

To establish pure spinal cord astroglia cultures, cells from P1 rat spinal cord were plated and grown in high serum conditions for 9 days before undergoing sequential shaking procedures to remove microglia and oligodendrocytes. After 3 more days in Ara-C supplemented medium, cells were replated and these spinal cord astroglia cultures consist of GFAP+ and vimentin+ cells which were also observed in the mixed cultures. There are no neurons or oligodendrocytes present in these cultures (data not shown).

Preliminary experiments were designed to examine whether UA elicits the secretion of soluble factors that contribute to the effects of UA. Astroglia cultures were grown in NB medium for 3 days and treated with UA or Locke's buffer. Conditioned medium (CM) from these cultures were collected 24 hours later. CM from the vehicle-treated group was designited as CM1. CM from UA treated group was CM2. The possible effects of CM1 and CM2 to rescue pure spinal cord neurons from glutamate toxicity were examined. Neither CM1 nor CM2 reduced the damage to neurons elicited by 10 μM glutamate (Figure 8), suggesting that soluble factors are not likely to be involved in mediating UA actions.

Further studies explored whether direct addition of astroglia to pure neuronal cultures could restore the effects of UA in protection from glutamate toxicity. These cells were grown for 5 days in SCM and then trypsinized and replated onto DIV 5 pure spinal cord neuron cultures. The medium for the combined cultures was changed to SCM. Twenty four hours later, the combined cultures were treated with glutamate followed by UA or vehicle addition. Examination of neuron numbers indicated that 100μM UA blocked the toxic effects of glutamate (Figure 9), suggesting that the presence of astroglia is essential for mediating the effects of UA.

EAAT-1 expressed by astroglia may play an important role in mediating the neuroprotective effect of UA

Astroglial cells have been reported to express excitatory amino acid transporters (EAATs), mainly EAAT-1 and EAAT-2. EAATs can remove extracellular glutamate and limit neuronal access to toxicity. Immunostaining studies indicated that EAATs are exclusively expressed by astroglial cells in our cultures. Interestingly, EAAT-1 is co-localized with GFAP+ astroglia and EAAT-2 is expressed by vimentin+ astroglia in the mixed spinal cord cultures (Figure 10 A). Furthermore, blockade of the EAAT activity by inhibitor L-Threohydroxy aspartate (THA) results in elimination of UA actions to reduce glutamate toxicity (Figure 10 B) These results suggest that EAATs expressed by astroglia may play an important role in mediating the neuroprotectiveeffects of UA.

Discussion

Glutamate toxicity plays a key role in the secondary phase of spinal cord injury (Hulsebosch, 2002). In fact, microdialysis studies indicate that glutamate concentrations rise sharply after injury and that the excessive level of glutamate damages neurons in the cord severely (Liu et al., 1991; Liu et al., 1999; Xu et al., 2005). In our experiments, cells were exposed to a brief, one-hour, treatment with glutamate. The possible roles of UA to protect neurons were tested against

500 μM glutamate, mimicking the concentration observed in rat spinal cord injury models (McAdoo et al., 1999). In spinal cord cultures grown in SCM, we demonstrated that UA, a ubiquitous anti-toxicant, protected spinal cord neurons against glutamate toxicity. A concentration of 100 μM UA reversed the cell loss elicited by treatment with 500μM glutamate (Figure 3). It should be noted that cultures grown in SCM are a mixed population. Together with neurons, oligodendrocytes and astroglial cells were present in these cultures (Figure 4). To examine whether these cell types are involved in UA actions on neuronal protection, pure spinal cord neuron cultures were developed, and the neuroprotective effect of UA was evaluated. Interestingly, UA did not protect spinal cord neurons against glutamate toxicity in these cultures (Figure 6). The lack of UA action on pure neurons suggests that glial cells are essential for reducing the damage to spinal cord neurons.

UA is generally considered to be a natural scavenger for peroxynitrite, a reactive oxygen species (ROS) that plays an important role in mediating tissue damage and cell loss in CNS injury and trauma (Keynes and Garthwaite, 2004). Thus, the role of UA as a compound against toxicity has been studied rigorously as a direct scavenger for ROS. Neuroprotection elicited by UA has been considered as a result of the reduction of ROS, which directly damage neurons. For example, studies using hippocampal neuronal cultures indicated that the neuroprotective effects of UA involve suppression of oxyradical accumulation (Yu et al., 1998). Moreover, previous findings by Scott and colleagues demonstrated that treatment of mouse spinal cord neuron cultures with UA blocked peroxynitrite toxicity (Scott et al., 2005). Furthermore, pre-treatment and concurrent treatment with UA reduced secondary damage in a mouse model of spinal cord injury (Scott et al., 2005). The ability of UA to dramatically block peroxynitrite toxicity was demonstrated in our study as well (Figure 7), suggesting that this pathway is regulated by the presence of UA. However, glutamate toxicity, which elicits a significant part of the secondary damage in spinal cord injury, could not be attenuated by UA in pure spinal cord neuron cultures, suggesting that peroxynitrite is not likely to be a major mediator in the

toxic signaling of glutamate and effects of UA in mixed cultured are not likely to be direct on neurons. Additionally, previous studies used a pre-treatment and concurrent treatment paradigm, which is an issue for therapeutic use of UA. Our studies show that UA can be used as a neuroprotective agent after injury, making it a viable treatment for SCI.

Our studies suggest an essential glial involvement in the effects of UA. In the SCM spinal cord cultures, neurons were very resistant to the toxic effects. There was only about 40% neuron loss after one-hour treatment with 500 μM glutamate (Figure 1), compared with almost total neuron elimination in the pure cultures (Figure 5). This is probably due to the presence of astroglial cells and their likely roles to protect neurons in these cultures. Previous reports have demonstrated that astroglial cells can reduce damage to neurons through distinct mechanisms. For example, early studies indicated that glutamate toxicity was much more potent in cortical neurons grown in an astrocyte-poor culture than those grown in an astrocyte-rich culture (Rosenberg et al., 1992). Astroglial cells have also been shown to secrete neuroprotective factors such as transforming growth factor beta (TGF-β; Dhandapani and Brann, 2003) and brain-derived neurotrophic factor (BDNF; Dougherty et al., 2000; Wu et al., 2004). Interestingly, adding conditioned media from UA-treated astroglial cultures did not rescue the neuroprotective effects in pure neuronal cultures (Figure 8), suggesting that in order for UA to act as a neuroprotectant, 1) astroglia must be physically present to rescue neurons from glutamate-induced death or 2) there must be some cross-talk between neurons and astroglia, possibly by secreted factors. In fact, the direct presence of astroglial cells significantly reduced glutamate damage (Figure 2 and Figure 5). Furthermore, the seeding of astroglial cells into pure spinal cord neuron culture re-instated the ability of UA to protect neurons from glutamate toxicity (Figure 9), suggesting that UA not only mediates its neuroprotective effects indirectly by acting on astroglia, but also requires the direct presence of astroglia in the cultures.

One likely candidate for mediating the effects of UA is the glutamate transporters. The two major types of glutamate transporters, EAAT-1 (Glial

Glutamate Transporter, GLAST) and EAAT-2 (Glial Glutamate Transporter-1, GLT-1), are primarily expressed in astroglial cells. These transporters have been shown to protect neurons from glutamate toxicity (Rothstein et al., 1993). Moreover, there are studies demonstrating that EAAT-1 and EAAT-2 are acutely up-regulated after spinal cord injury (Vera-Portocarrero et al., 2002). Our work employed an EAAT inhibitor, THA, to examine the possible involvement of these transporters in the neuroprotective effects of UA. Treatment with THA blocked the effects of UA, suggesting that EAATs play a key role in mediating the actions of UA.

The roles of astroglia after spinal cord injury have been studied rigorously. Astroglia, especially reactive astrocytes, have been found to form the glia scar, generally considered as a major impediment to axon regeneration (Liuzzi and Lasek, 1987; Rudge and Silver, 1990). However, more recent studies indicate that astrocytes may protect tissue and preserve function after SCI (Faulkner et al., 2004; Silver and Miller, 2004). In our study, the different levels of damage elicited by glutamate in mixed and pure neuron cultures suggest a beneficial role played by astroglia in the acute phase of SCI. Our work reveals an intriguing prospect that astroglia mediate the effects of UA to reduce glutamate-elicited damage to neurons, casting new insight into possible roles astroglia can play in the anti-excitotoxic process after trauma. Furthermore, our results suggest that UA can act as a neuroprotective agent after glutamate exposure, further supporting its use as a therapeutic agent after SCI.

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treated with Sin-1 (250 μM) for 1 hour. (A) UA (100 μM) was added with and after glutamate treatments. (B) UA (100 μM) was only after glutamate treatments. Cells were fixed after 24 hours and numbers of neurons that survived (MAP-2+) were counted. Results were derived from 2 independent experiments (n=6). ***p<0.001 by parametric ANOVA followed by Bonferroni Multiple Comparisons Test.

Figure 8. Condition medium (CM) from astroglial cultures does not reduce glutamate toxicity to pure spinal cord neurons. CM was collected from pure spinal cord astroglial cultures grown in NB and treated with UA or Locke's buffer. CM1 was from Locke's buffer treated group. CM2 was from UA treated group (containing UA). Pure neuron cultures were treated with glutamate (10 μM) for 1 hour and medium was changed to CM. Cells were fixed after 24 hours and numbers of neurons that survived (MAP-2+) were counted. Results were derived from 2 independent experiments (n=6). ***p<0.001 by parametric ANOVA followed by Bonferroni Multiple Comparisons Test.

Figure 9. Re-plating of astroglial cells in pure neuron cultures reinstates the effects of UA in reducing glutamate toxicity. Pure astroglial cultures were established as described in the Methods and Materials. These cells were trypinized and re-plated in pure spinal cord neuron cultures grown in SCM for 5 days. After 24 hours, cells were exposed to glutamate (50 μM) for 1 hour and UA (100 μM) was added after the medium was changed. Cells were fixed after 24 hours and numbers of neurons that survived (MAP-2+) were counted. Results were derived from 2 independent experiments (n=6). ***p<0.001 by parametric ANOVA followed by Bonferroni Multiple Comparisons Test.

Figure 10. EAAT-1 is expressed by GFAP+ astroglia, EAAT-2 is expressed by vimentin+ astroglia, and treatment with the EAAT inhibitor THA blocks the effects of UA to protect neurons against glutamate toxicity. (A) Mixed cultures derived from spinal cords of E16 rats were fixed on DIV 7. Cells were double labeled for

EAAT-1 and GFAP or EAAT-2 and vimentin. Scale bar, 50 μm. (B) THA (50 μM) was added to DIV 6 mixed cultures one hour prior to a one-hour exposure to glutamate (50 μM). THA (50 μM) and UA (100 μM) were added when the medium was changed. Cells were fixed after 24 hours and numbers of neurons that survived (MAP-2+) were counted. Results were derived from 2 independent experiments (n=6). ***$p<0.001$ by nonparametric ANOVA followed by Dunn's analysis comparing treated groups with control.

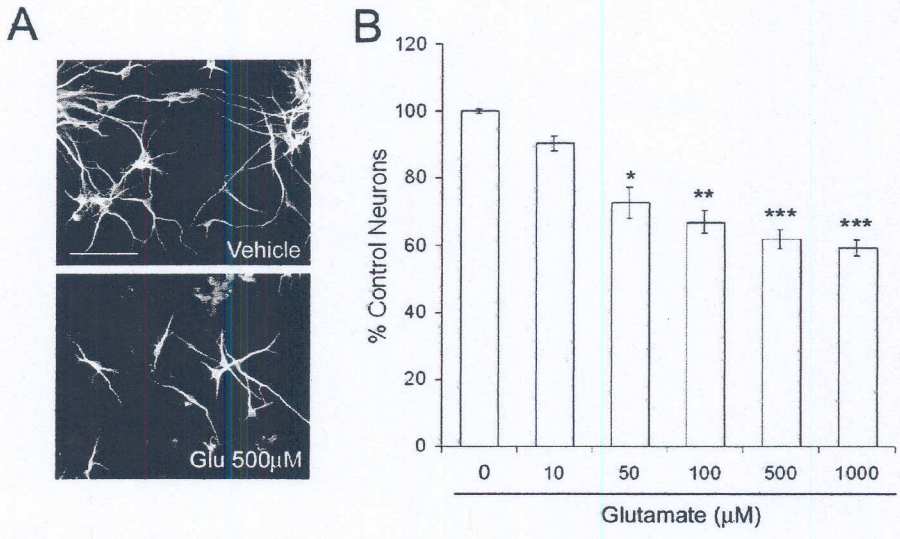

Figure 1. Glutamate induces loss of spinal cord neurons in a dose-dependent manner. Spinal cord neurons were grown in SCM for 6 days before being treated with glutamate for 1 hour. Cells were fixed after 24 hours and stained with anti-MAP-2 antibody. (A) Control cultures and cells treated with 500mM glutamate were illustrated. (B) Numbers of neurons that survived (MAP-2+) were counted. Increasing concentrations of glutamate resulted in more significant loss of spinal cord neurons. Results were derived from 3 independent experiments (n=7). * $p<0.05$, *** $p<0.001$ by nonparametric ANOVA followed by Dunn's analysis for multiple comparisons comparing glutamate treated groups with control. Scale bar, 50 um.

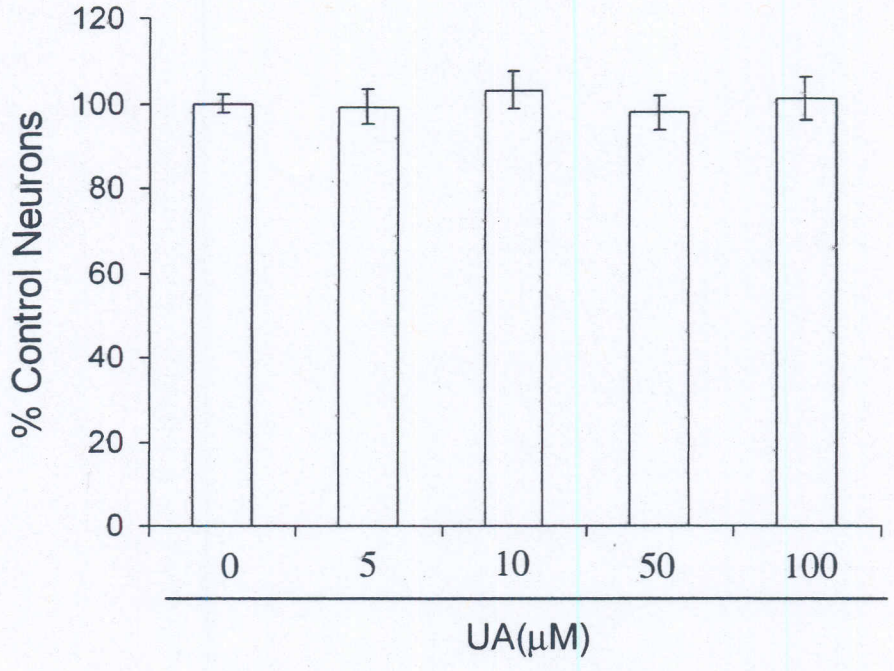

Figure 2. UA does not affect survival of spinal cord neurons. Day 6 spinal cord cultures grown in SCM were treated with UA (5-100mM) for 24 hours before being fixed and stained with anti-MAP-2. MAP-2+ cell numbers were counted and the results suggested that UA did not alter neuron survival. Results were derived from 2 independent experiments (n=6).

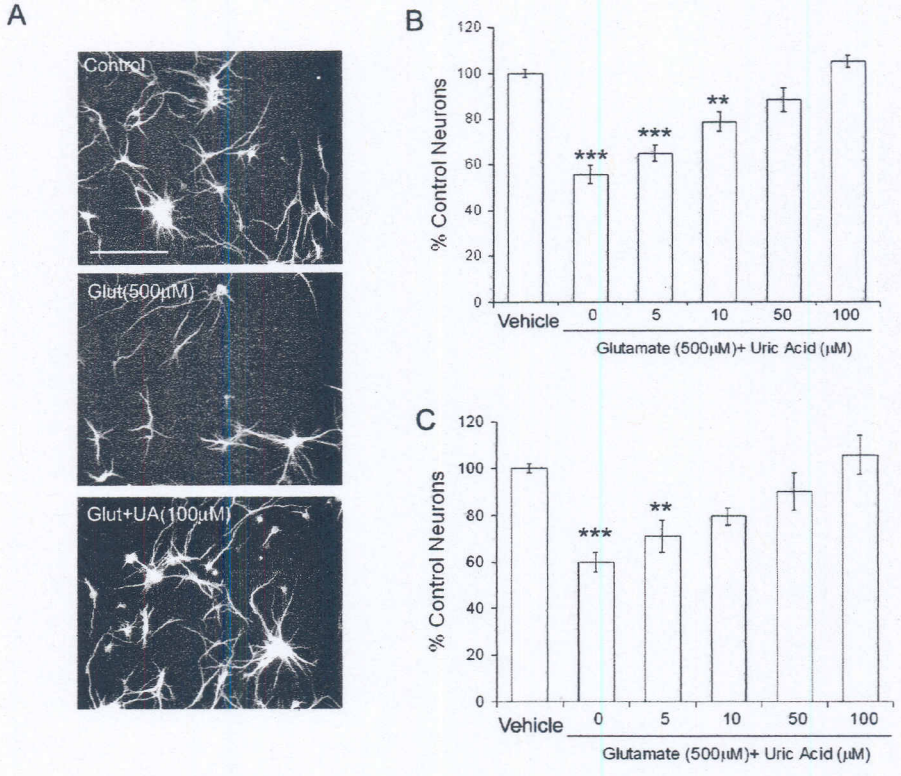

Figure 3. UA blocks glutamate toxicity to spinal cord neurons. Spinal cord neurons were grown in SCM for 6 days before being treated with glutamate for 1 hour, with or without the presence of UA. Cells were fixed after 24 hours and stained with anti-MAP-2 antibody. (A) Control cultures, cells treated with 500mM glutamate, and cells treated with 500mM glutamate and 100mM UA (add together) are illustrated. (B) When glutamate and UA were added together, UA blocked glutamate toxicity. Results were derived from 6 independent experiments (n=12). (C) When UA was added after glutamate treatment, it had similar effects to reverse glutamate damage. Results were derived from 3 independent experiments (n=9). * $p<0.05$, ** $p<0.01$, *** $p<0.001$ by nonparametric ANOVA followed by Dunn's analysis for multiple comparisons using vehicle as control. Scale bar, 50 mm.

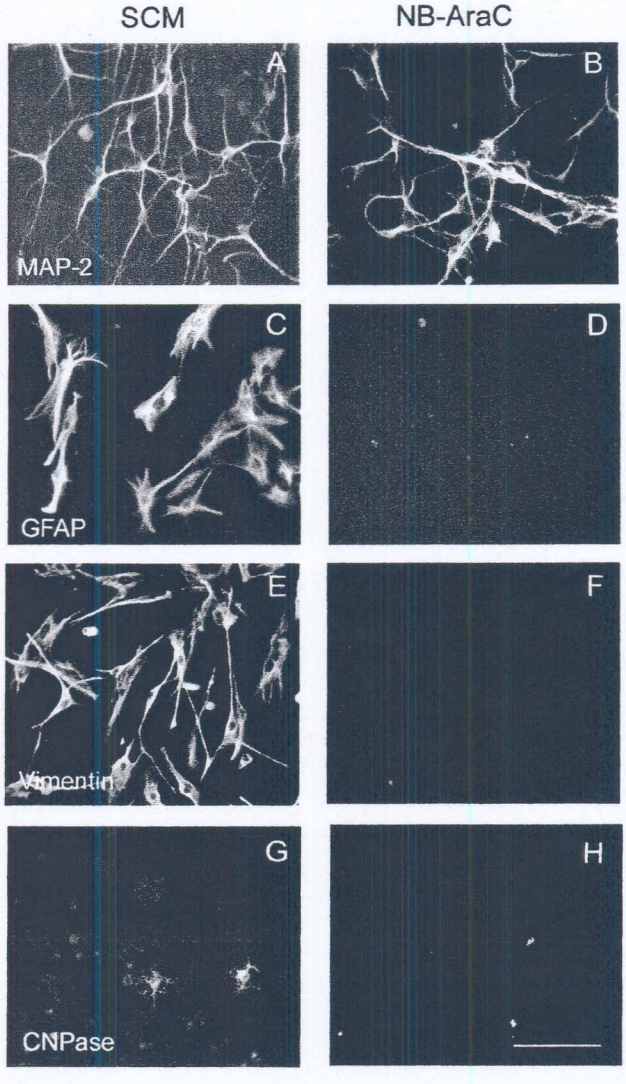

Figure 4. Characterization of cell types present in cultures grown in SCM and cultures grown in NB and treated with Ara-C. The two types of spinal cord cultures were grown for 7 days before being fixed and stained with MAP-2, marker for neurons, or GFAP, marker for astrocytes, or CNPase, marker for oligodendrocytes. Cells grown in SCM were mixed cultures with all three cell types present. Cells grown in NB and treated with Ara-C were a pure neuronal population. Scale bar, 50 mm.

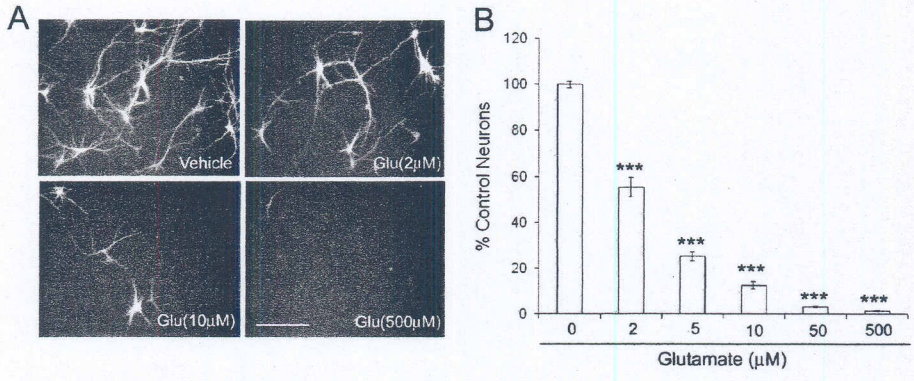

Figure 5. Dose-dependent cell loss in pure spinal cord neuron cultures to glutamate toxicity. Pure spinal cord neuron cultures were established, and after 6 days, these cells were treated with glutamate for 1 hour. Cells were fixed after 24 hours and stained with anti-MAP-2 antibody. (A) Control cultures and cells treated with 2, 10, and 500 mM glutamate are illustrated. (B) Numbers of neurons that survived (MAP-2+) were counted. Increasing concentrations of glutamate resulted in a more significant loss of spinal cord neurons. Results were derived from 3 independent experiments (n=7). ***p<0.001 by nonparametric ANOVA followed by Dunn's analysis comparing glutamate treated groups with control. Scale bar, 50 mm.

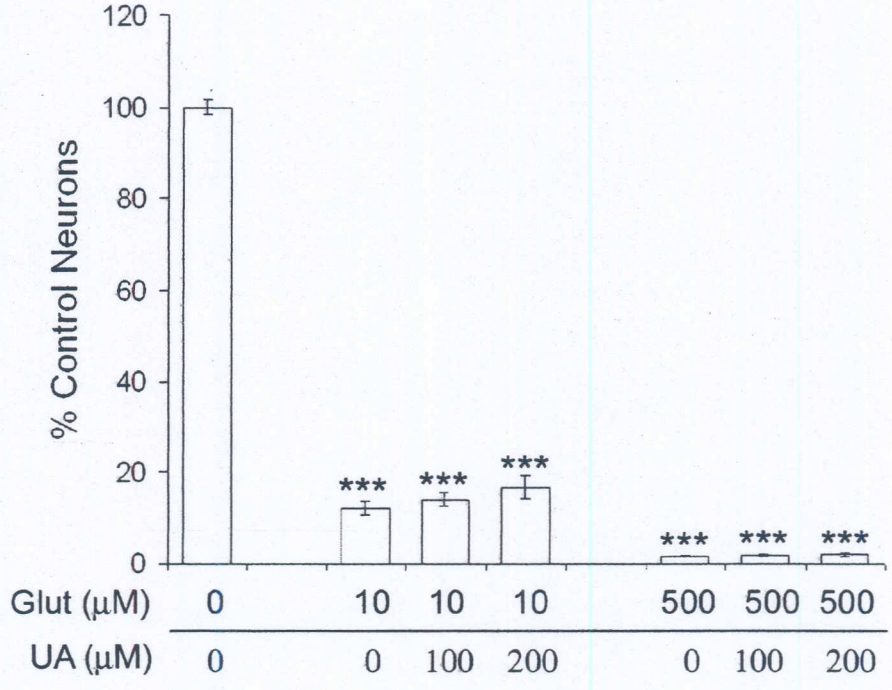

Figure 6. UA does not decrease glutamate toxicity in pure spinal cord neuron cultures. Six days after plating, pure spinal cord neuron cultures were treated with glutamate (10 or 500 mM) for 1 hour. UA (100 or 200 mM) was added with and after glutamate treatments. Cells were fixed after 24 hours and numbers of neurons that survived (MAP-2+) were counted. Results were derived from 2 independent experiments (n=6). ***p<0.001 by nonparametric ANOVA followed by Dunn's analysis for multiple comparisons comparing treated groups with control.

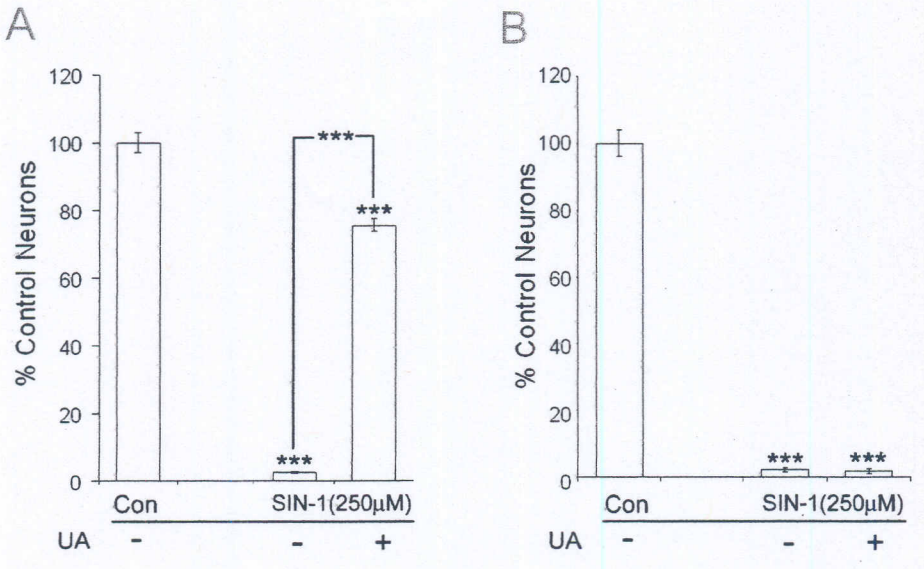

Figure 7. UA reduces damages elicited by Sin-1 treatment in pure spinal cord neuron cultures. Six days after plating, pure spinal cord neuron cultures were treated with Sin-1 (250 mM) for 1 hour. (A) UA (100 mM) was added with and after glutamate treatments. (B) UA (100 mM) was only after glutamate treatments. Cells were fixed after 24 hours and numbers of neurons that survived (MAP-2+) were counted. Results were derived from 2 independent experiments (n=6). ***p<0.001 by parametric ANOVA followed by Bonferroni Multiple Comparisons Test.

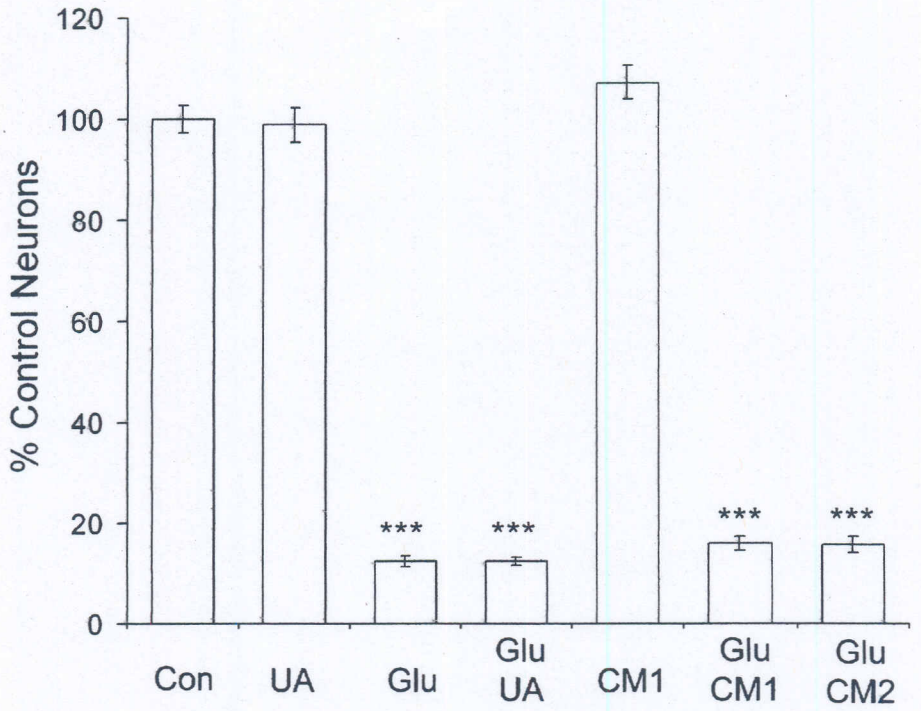

Figure 8. Condition medium (CM) from astroglial cultures does not reduce glutamate toxicity to pure spinal cord neurons. CM was collected from pure spinal cord astroglial cultures grown in NB and treated with UA or Locke's buffer. CM1 was from Locke's buffer treated group. CM2 was from UA treated group and it contained UA. Pure neuron cultures were treated with glutamate (10 mM) for 1 hour and medium was changed to CM. Cells were fixed after 24 hours and numbers of neurons that survived (MAP-2+) were counted. Results were derived from 2 independent experiments (n=6). ***p<0.001 by parametric ANOVA followed by Bonferroni Multiple Comparisons Test.

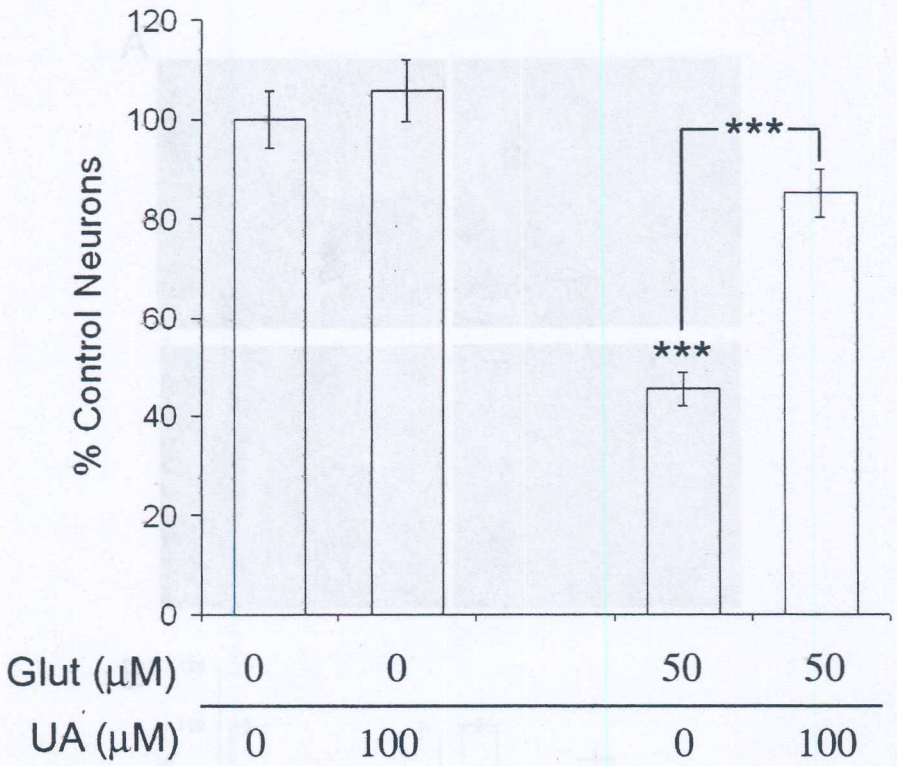

Figure 9. Re-plating of astroglial cells in pure neuron cultures reinstates the effects of UA in reducing glutamate toxicity. Pure astroglial cultures were established as described in the Methods and Materials. These cells were trypinized and re-plated in pure spinal cord neuron cultures grown in SCM for 5 days. After 24 hours, cells were exposed to glutamate (50 mM) for 1 hour and UA (100 mM) was added after the medium was changed. Cells were fixed after 24 hours and numbers of neurons that survived (MAP-2+) were counted. Results were derived from 2 independent experiments (n=6). ***p<0.001 by parametric ANOVA followed by Bonferroni Multiple Comparisons Test.