FINAL NARRATIVE REPORT
INDIVIDUAL RESEARCH GRANT

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Name of Institution: Rutgers, the State University of New Jersey
Grant Title: The Role of Cypin in Protecting Spinal Cord Neurons from Glutamate-Induced Toxicity
Grant Number: 03-3024-SCR-E-0
Grant period: 06/15/03-06/14/06
Date of Submission: 08/18/06

1. Original aims of the project:

Aim I. Does uric acid protect cultured spinal cord neurons from glutamate- and peroxide-induced toxicity?

Aim II. Can cypin increase neurite outgrowth or branching in the spinal cord? (Changed from "Can cypin block glutamate signaling?" in year 2 renewal).
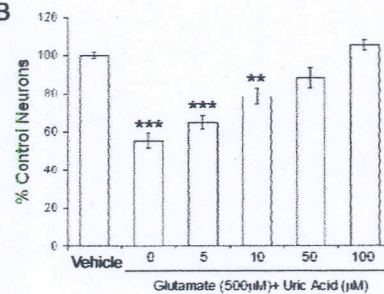
2. Project successes:

As a new investigator to the field of SCI, I am excited to report that our work funded by the NJCSCR has highly successful. We have submitted a manuscript on our findings for publication and will present our work (published in abstract form) at the Society for Neuroscience 2006 meeting in Atlanta, Georgia. I will summarize our results here.
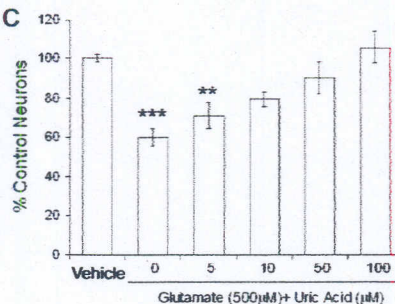
Aim I:

UA protects spinal cord neurons from glutamate-induced toxicity. Glutamate is the major physiological agent that mediates cell death after spinal cord injury. 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. Thus, we asked whether UA can protect spinal cord neurons from glutamate-induced death. 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,

Figure 1. 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 500µM glutamate, and cells treated with 500µM glutamate and 100µM 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 µm.

glutamate-promoted neuronal cell death was abolished (Figure 1A). Most importantly, treatment of UA solely after the termination of glutamate exposure resulted in similar neuroprotection (Figure 1 B). These results suggest that UA can act to protect neurons after glutamate exposure in culture.
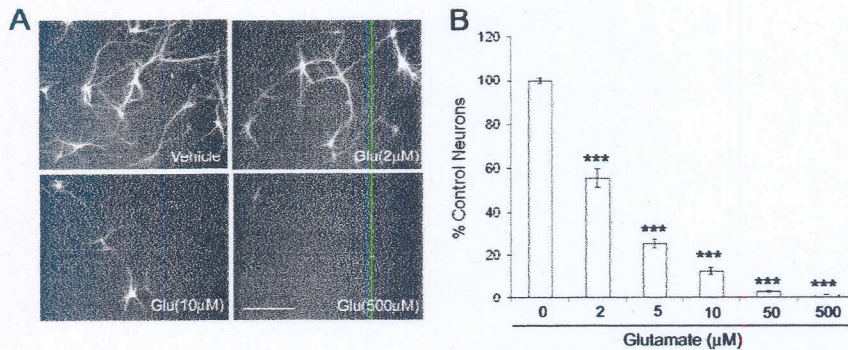
A / B

Figure 2. 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 μM 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 μm.
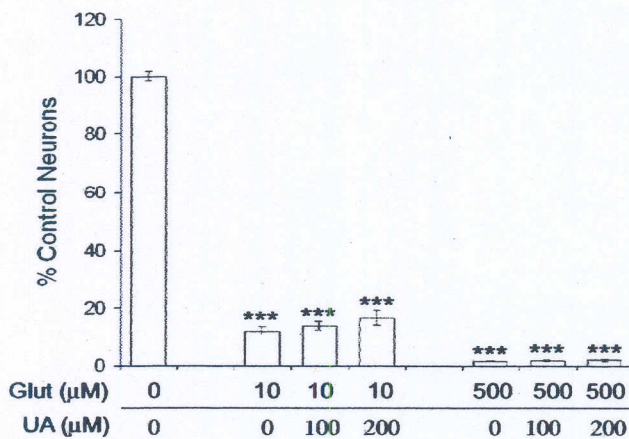

Figure 3. UA does not decrease glutamate toxicity in pure spinal cord neuron cultures. Six days after plating, pure spinal cord neuron cultures were treated with glutamate (10 or 500 μM) for 1 hour. UA (100 or 200 μM) was added with and after glutamate treatments. Cells were fixed after 24 hours and 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.

UA cannot protect spinal cord neurons from glutamate toxicity in pure neuron 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. 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 2), and 500 μM glutamate essentially eliminated all neurons (Figure 2), 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
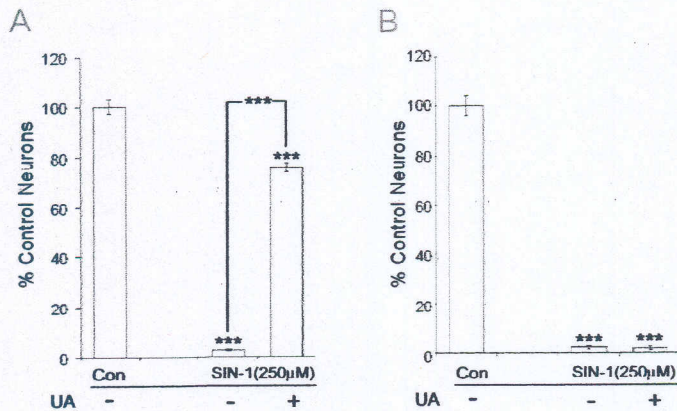
Figure 4. UA reduces damages elicited by Sin-1 treatment in pure spinal cord neuron cultures. Six days after plating, pure spinal cord neuron cultures were treated with Sin-1 (250 μM) for 1 hour. (A) UA (100 μM) was added with and after glutamate treatments. (B) UA (100 μM) was only after glutamate treatments. 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.
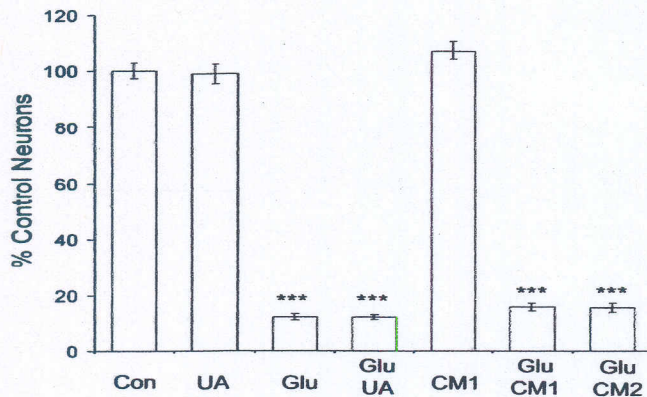
Figure 5. Conditioned medium (CM) from astroglial cultures does not reduce glutamate toxicity to pure spinal cord neurons. CM was collected from pure spinal cord astroglial cultures grown in NB and treated with UA or Locke's buffer. CM1 was from Locke's buffer treated group. CM2 was from UA treated group (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.

against glutamate toxicity (Figure 3). 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 4 A). However, when UA was added after Sin-1, it did not elicit a reversal of toxicity (Figure 4 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 designated 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 5), suggesting that soluble factors are not likely to be involved in mediating UA actions.

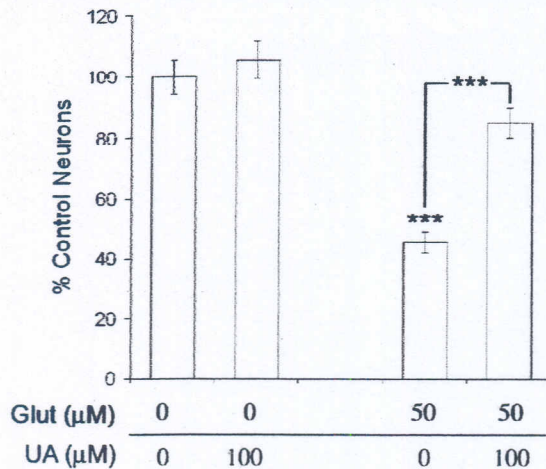| Glut (μM) | 0 | 0 | 50 | 50 |
| UA (μM) | 0 | 100 | 0 | 100 |

Figure 6. 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.

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 6), 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 7 A). Furthermore, blockade of the EAAT activity by inhibitor L-Threohydroxy aspartate (THA) results in elimination of UA actions to reduce glutamate toxicity (Figure 7 B). These results suggest that EAATs expressed by astroglia may play an important role in mediating the neuroprotective effects of UA.

Thus, we have identified a novel mechanism by which neurons may be protected from toxicity immediately after SCI.
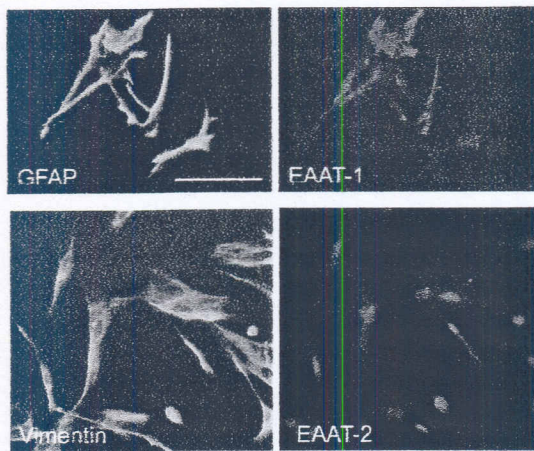
Figure 7. EAAT-1 is expressed by GFAP+ astroglia, EAAT-2 is expressed by vimentin+ astroglia, and treatment with the EAAT inhibitor THA blocks the effects of UA to protect neurons against glutamate toxicity. (A) Mixed cultures derived from spinal cords of E16 rats were fixed on DIV 7. Cells were double 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.
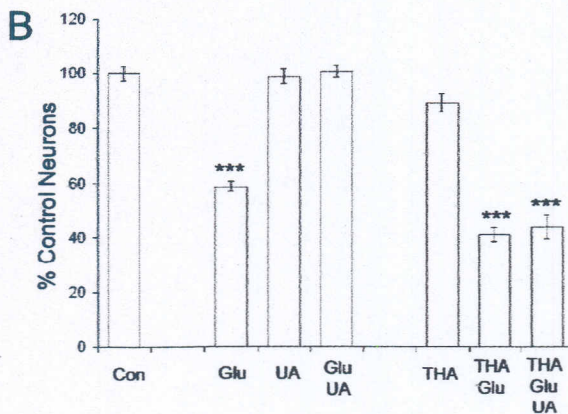
Aim II:

The developing spinal cord expresses cypin. Since one of our hypotheses is that cypin may act in spinal cord neurons to protect spinal cord neurons from injury by increasing uric acid production and to increase dendrite outgrowth and/or branching, cypin should be expressed in the spinal cord during development or injury. Our preliminary data suggest that cypin protein is indeed expressed in developing spinal cord (Figure 8). In collaboration with Dr. Crista Adamson in the W. M. Keck Center for Collaborative Neuroscience at Rutgers University, we are currently examining whether cypin protein is expressed in damaged spinal cord. As seen in Figure 9, it appears that cypin protein is upregulated in damaged adult spinal cord. Furthermore, cypin is expressed in control spinal cord (Figure 9). Thus, our cultures of developing spinal cord neurons will help us to understand putative neuroprotective and regenerative mechanisms.

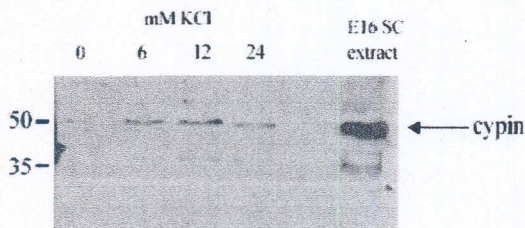
Figure 8. Treatment with KCl increases cypin protein expression in spinal cord cultures. Extracts from spinal cord cultures treated with KCl from DIV 7-10 or from E16 rats were subjected to SDS-PAGE. Cypin protein expression was determined by Western blotting.

Cypin is expressed in cultures of spinal cord neurons from E16 rats. We find that our cultures express cypin protein (Figure 8). This is an important finding since culturing neurons may result in downregulation of proteins normally expressed in neurons in the intact organism. We have found that cypin is expressed in
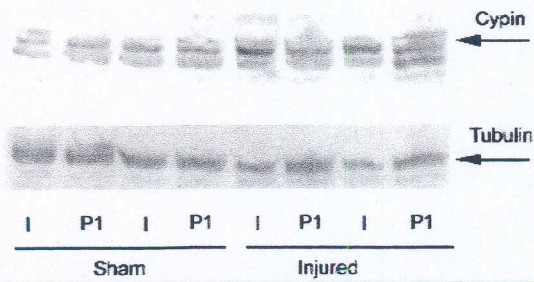
Figure 9. Cypin levels in injured and uninjured rats. Spinal cord extracts (10 µg/lane) from rats subjected to SCI using the NYU Impactor (10 gram weight dropped from 25 mm) or to sham SCI were analyzed by SDS-PAGE and immunoblotting for the presence of cypin and tubulin (control for amount of protein loaded). I = impact site and P1 = 5 mm away from the impact site.

spinal cord neurons (data not shown) and are currently characterizing whether cypin protein is enriched in a subset of these neurons.

Activity increases cypin levels in spinal cord cultures. As we have described for hippocampal neurons (Akum et al., 2004), treatment with increasing amounts of KCl results in increased expression of cypin in hippocampal neurons. We propose that activity could also increase cypin levels in spinal cord neurons and that we could use this paradigm to increase cypin levels to protect spinal cord neurons from glutamate-, manganese- and/or peroxide-induced toxicity. Interestingly, activity does increase cypin protein levels in spinal cord cultures (Figure 8). Thus, we can use KCl to increase cypin levels concurrent with or after treating with glutamate and assess cypin's role in neuroprotection.

3. Project challenges:

We have made quite a bit of progress on our proposed work. Our hypotheses changed since the original proposal was based on reports that uric acid acts as an anti-oxidant to protect neurons, and we found that this is not uric acid's main mode of action. Overall, the challenges were minor and are considered within the normal realm of scientific progress.

4. Implications for future research and/or clinical treatment:
The identification of uric acid action will give us targets for drug therapy to treat SCI. Furthermore, Hooper and colleagues at Thomas Jefferson University showed that uric acid may act to restore function after SCI in mice.

5. Plans to continue this research:
We would like to continue this research; however, we applied for more funds from NJCSCR and have been asked to revise our application. Since NIH funding stands at below 10% right now, we would need this funding to generate more data on the mechanism of uric acid action before we apply to NIH.

6. Publications emerging from this research:
Abstract: Effects of uric acid in protecting spinal cord neurons from glutamate toxicity. Yangzhou Du, Christopher Chen, Yuval Eisenberg, Bonnie L. Firestein. Society for Neuroscience Annual meeting 2006. Atlanta, GA.

Manuscript submitted and in review: Du, Y., Chen, C.P., Tseng, C.Y., Eisenberg, Y. and Firestein, B.L. (2006) Astroglia-mediated effects of uric acid to protect spinal cord neurons from glutamate toxicity. Submitted to *Glia*.