Published in final edited form as:

Neuroscience. 2008 June 2; 153(4): 1034–1047. doi:10.1016/j.neuroscience.2008.02.065.

Glutamate-Induced Losses of Oligodendrocytes and Neurons and Activation of Caspase-3 in the Rat Spinal Cord

Guo-Ying Xu^a, Song Liu^b, Michael G. Hughes^a, and David J. McAdoo^{a,b}

^a Department of Neuroscience and Cell Biology, University of Texas Medical Branch 301 University Blvd., Galveston, TX 77555-1043

^b Department of Biochemistry and Molecular Biology, University of Texas Medical Branch 301 University Blvd., Galveston, TX 77555-1043

Abstract

The toxicity of released glutamate contributes substantially to secondary cell death following spinal cord injury (SCI). In this work, the extent and time courses of glutamate-induced losses of neurons and oligodendrocytes are established. Glutamate was administered into the spinal cords of anesthetized rats at approximately the concentration and duration of its release following SCI. Cells in normal tissue, in tissue exposed to artificial cerebrospinal fluid and in tissue exposed to glutamate were counted on a confocal system in control animals and from 6 h to 28 days after treatment to assess cell losses. Oligodendrocytes were identified by staining with antibody CC-1 and neurons by immunostaining for NeuN or Neurofilament H. The density of oligodendrocytes declined precipitously in the first 6 h after exposure to glutamate, and then relatively little from 24 h to 28 days post-exposure. Similarly, neuron densities first declined rapidly, but at a decreasing rate, from 0 h to 72 h post-glutamate exposure and did not change significantly from 72 h to 28 days thereafter. The nuclei of many cells strongly and specifically stained for activated caspase-3, an indicator of apoptosis, in response to exposure to glutamate. Caspase-3 was localized to the nucleus and may participate in apoptotic cell death. However, persistence of caspase-3 staining for at least a week after exposure to glutamate during little to no loss of oligodendrocytes and neurons demonstrates that elevation of caspase-3 does not necessarily lead to rapid cell death. Beyond about 48 h after exposure to glutamate, locomotor function began to recover while cell numbers stabilized or declined slowly, demonstrating that functional recovery in the experiments presented involves processes other than replacement of oligodendrocytes and/or neurons.

Keywords

apoptosis; caspase-3; nuclear localization; excitotoxicity; microdialysis; spinal cord injury

Cell death by secondary processes contributes substantially to the crippling that follows spinal cord injury (SCI). Loss of hindlimb function following SCI reflects damage to long axons (Blight, 1983; Noble and Wrathall, 1989; Basso et al., 1996; Cao et al., 2005; Kloos et al., 2005), to central pattern generating circuitry (Magnuson et al., 1999, 2005) and/or to pertinent motor neurons (Collazos-Castro et al., 2005), depending on the location of the injury. Impairment of hindlimb movements by damage anterior to the motor neurons for those limbs reflects reduced conduction in long descending and ascending spinal cord axons (Blight, 1983;

Noble and Wrathall, 1989; Basso et al., 1996; Kloos et al., 2005) due to damage to those axons and their myelination (Waxman, 1989). Concentrations of glutamate rapidly rise and fall following SCI (Panter et al., 1990; Liu et al., 1991; 1999; McAdoo et al., 1999). Glutamate toxicity contributes substantially to secondary damage following neurotrauma (Faden and Simon, 1988; Faden et al., 1988, 1989). Administration of glutamate into the rat spinal cord at the concentrations and duration of its release following SCI significantly reduces the numbers of neurons (Liu et al., 1991; 1999) and oligodendrocytes (Xu et al., 2004) present at 24 h post-SCI in the area of infusion and impairs locomotor function (Xu et al., 2005). Treatment with glutamate also reduces the amplitudes of compound action potentials and damages oligodendrocytes in isolated spinal dorsal columns (Li and Stys, 2000), phenomena that likely contribute to locomotor impairments stemming from SCI.

Advancing our understanding of SCI and improving its treatment requires better defining the mechanisms underlying the development of functional deficits following SCI and their time courses. To this end, we characterized further the effects on neurons and oligodendrocytes of administering glutamate into the spinal cord at the approximate concentrations and time courses of its release following SCI. Kainic acid has been applied to the spinal cord to demonstrate the effect of stimulating AMPA/kainate receptors (Nottingham and Springer, 2003). However, we applied glutamate rather than exogenous agonists because, since exogenous glutamate agonists target specific receptor sub-types, application of such an agonist would less fully reproduce the actions of glutamate. Furthermore, uptake reduces concentrations of administered glutamate (Mangano and Schwarz, 1983; McBean and Roberts, 1985), but not the concentrations of exogenous agonists, another factor that administering glutamate itself should preserve. The substantial early decreases in the numbers of oligodendrocytes and neurons in response to glutamate administration correlate with previously observed early loss of function in response to the same treatment, but beyond day 2 functional recovery occurs during some continuing decline or stabilization of the numbers of those cells (Xu et al., 2005). Production of apoptotic features (response to an apoptosis-specific ELISA, TUNEL staining and caspase-3 activation) by exposure to glutamate is also described.

EXPERIMENTAL PROCEDURES

Animal preparation and exposure to glutamate

Male Sprague-Dawley rats (200–250 g) from Harlan-Sprague Dawley, Inc. (Houston, TX) were anesthetized with pentobarbital (50 mg/kg i.p.). Anesthesia was considered complete when there was no flexor withdrawal in response to noxious foot pinch. Following the onset of anesthesia, the back was disinfected and shaved, the shaved area was washed with betadine, and the cord was exposed at spinal cord segment T10 by removal of muscle followed by a laminectomy of vertebrae T9-T10. During anesthesia and microdialysis, body temperature was maintained with a heating pad and the exposed cord was covered with warm mineral oil to maintain its temperature. To administer glutamate, a microdialysis fiber was inserted laterally through the cord as described below at cord segment T10. These fibers were prepared by coating, excluding a 2 mm dialysis zone, hollow porous fibers (Spectrum Industries) with a thin layer of silicon rubber (Dow Chemical 3140 RTN; the outer fiber diameter after coating = 220–240 μm), as described previously (Sorkin et al., 1988; Liu et al., 1999; Xu et al., 2005). After fiber insertion, artificial cerebrospinal fluid (ACSF) was pumped through the fiber at a flow rate of 2 $\mu\text{l}/\text{min}$ for 60 min to allow glutamate release caused by insertion damage to subside. The composition of ACSF in mM was: 151.1 Na^+ , 2.6 K^+ , 0.9 Mg^{2+} , 1.3 Ca^{2+} , 122.7 Cl^- , 21.0 HCO_3^- , 2.5 HPO_4^{2-} and 3.87 glucose. Before use, all solutions administered through the fiber were bubbled with a 5% CO_2 /95% O_2 mixture and their pH was adjusted to 7.2. ACSF or glutamate dissolved in ACSF was administered through the fiber for 90 min, the time that glutamate is substantially elevated following SCI (McAdoo et al., 1999). Solutions were

administered by a person blind to the identity of the treatment. The glutamate concentration immediately outside the microdialysis fiber has been estimated to be 8X lower than that inside the fiber (Liu et al., 1999; Xu et al., 2004), and this concentration rapidly decreases further with increasing distance from the fiber (McAdoo et al., 1999). Thus the glutamate concentration to which the tissue was exposed was 8X or more below the concentration inside the fiber in all experiments (see below).

Following agent administration, the wound was closed and the animal allowed to awaken and returned to its cage. Bladders were manually expressed twice daily until control returned, usually 3–5 days after exposure to glutamate. After glutamate administration, the microdialysis fiber was cut off just outside the cord and left in place to enable location of its track upon sectioning. Fibers were still present in the cord at 7 days post glutamate exposure, but disappeared by 28 days after this treatment.

Immunocytochemistry

For immunocytochemistry, animals were re-anesthetized after the elapse of the selected post-treatment times, transcardially perfused with 100 ml of heparinized 0.9% saline and then with 1000 ml of cold 4% aqueous formalin solution (pH 7.4) to remove blood and fix the tissue. Next the portion of the cord containing the administering microdialysis fiber was removed. Control tissue was similarly taken from the corresponding region of the spinal cords of normal rats. After overnight incubation in 30% sucrose solution, the sections were post-fixed in 4% formalin at room temperature for 2 h and embedded in O.C.T. compound embedding medium (Miles, Elkhart, IN) and stored at -70°C . The tissue block containing the site of fiber insertion was cut into 10 μm transverse sections on a cryostat. These sections were mounted on gelatin-coated microscope slides, dried, and cover slipped with mounting medium for fluorescence microscopy (H-100, Vector Lab). The mounted sections were then with three 10 min rinses of phosphate-buffered saline (PBS) followed by blocking with 0.15% Triton X-100 + 5% normal goat serum (NGS) in 0.1 mM PBS for 30 min.

Sections were exposed to a solution containing a clone of a mouse monoclonal antibody to CC-1 (Oncogene Research Products), a specific oligodendrocyte marker (Bhat et al., 1996; Crowe et al., 1997; Rosenberg et al., 1999) (diluted 1:200), a polyclonal antibody specific to Neurofilament H (rabbit, lysine-serine-proline repeat; diluted 1:1000; AB1991, Chemicon) for 4 h at room temperature or an antibody to NeuN (Chemicon, mouse monoclonal, anti-rat/human; diluted 1:200 and kept overnight at 4°C).

Some tissue sections were exposed to an antibody to activated (cleaved) caspase-3 plus an antibody to NeuN or to CC-1 (R&D Systems, rabbit-anti-mouse/human, diluted 1:1000). Antibodies were diluted in 0.1 M PBS + 0.15% Triton X-100 + 1% NGS. The next day the sections were incubated with a 1:200 dilution of either goat anti-mouse IgG conjugated with Alexa Fluor 488 to stain for CC-1 or NeuN and/or the same dilution of goat anti-rabbit IgG conjugated to Alexa Fluor 568 to stain for Neurofilament H or activated caspase-3. After exposure for 4 h, slides were rinsed 5X in 0.1 mM PBS, cover slipped and stored in a freezer. The stained sections were thawed and photographed using a BioRad Radiance 2100 confocal analysis system combined with a Nikon Eclipse E 800 microscope and software. Images were collected simultaneously from two channels, individual excitation wavelengths being 488 nm for green (oligodendrocytes or NeuN stained neurons) and 568 nm for red (staining for Neurofilament H or caspase-3). Specificities of primary antibodies were demonstrated by an absence of staining when those antibodies were omitted from the staining procedure.

A NeuroTACSTM2 TDT-fluor in situ Apoptosis Detection Kit (Trevigen, Inc., catalog number 4812–30K) was employed to label cells for apoptosis. Following treatment, animals were sacrificed by cardiac perfusion with 4% formalin, and tissue from the region of interest

removed. This tissue was fixed in 4% formalin for 3 h, placed in 30% sucrose overnight and cut into 10 μm frozen sections. The sections were mounted on glass slides with mounting media, coverslipped and permeabilized with Cytonin IHCTM (Trevigen) at room temperature for 1 h in a humidity chamber followed by washing in PBS (7.5 mM Na_2HPO_4 , 2.5 mM NaH_2PO_4 , 0.145 mM NaCl). Sections were then incubated overnight in a humidity chamber in a CC-1 primary antibody solution (Oncogene Research Products; 1:200 dilution in Cytonin IHCTM) at 4 °C. The sections were then washed three times in PBS, incubated at room temperature for 4 h in an IgG Alexa Fluor 568-tagged anti-mouse secondary antibody diluted 1:200 in Cytonin IHCTM. The tissue was then subjected to three 5 min washings in PBS and immersed in quenching solution for 5 min at room temperature. Sections were then placed in 1X TdT labeling buffer for 5 min, covered with 50 μl of Labeling Reaction Mix (diluted 1:50), incubated at 37°C for 1 h in a humidity chamber and immersed in a 1X TdT Stop Buffer for 5 min at room temperature. Samples were then washed twice for 2 min in PBS at room temperature and incubated under 50 μl of Strept-Fluor Solution (diluted 1:50) for 20 min at room temperature in the dark. Labeling intensity was followed under a microscope to determine the optimum incubation period. Samples were then washed twice in PBS, immersed in diaminobenzidine solution for 7 min and finally twice in deionized water.

Location of cell counts

The fiber was inserted directly beneath the entry point of the dorsal root of spinal nerve T10 in all experiments. Areas were counted in transverse sections of the cord, the counting area being located relative to the microdialysis fiber track as follows. Areas sampled were located at the same distances laterally and ventrally from the fiber track in all experiments (Figure 1A) to ensure comparison of areas of comparable exposure because of steep gradients of administered glutamate around the fiber. Rostral locations were 100 μm from the edge of the fiber track (Figure 1B), except when a greater distance is specified. All cell counts were made in the ventral right quadrant of the cord at the stated distances rostral to the track of the administering fiber. Neurons were counted in the gray matter in areas located dorsoventrally and laterally as indicated in Figure 1A, and oligodendrocytes were counted in the adjacent white matter in the ventral area of the lateral funiculus (Figure 1A). All bright green cells in the areas selected were counted as oligodendrocytes when stained for CC-1, or as neurons when the staining was for NeuN. Some sections were also stained for activated caspase-3. All pictures were taken under computer control such that the location of the focal planes for illumination with the different lasers coincided.

Fluorescing cells were counted in five adjacent sections in 105 μm X 105 μm (0.011 mm^2) areas beginning at 100 μm rostral from the rostral edge of the fiber track unless a distance further rostral is indicated. The 100 μm distance was at the near surface of the eleventh transverse 10 μm section from the rostral edge of the fiber track (Figure 1B). The areas counted extended ventrally from the level of the bottom edge to the fiber track to 105 μm below that level. The counts for the five sections were averaged to obtain one density value per cell type per animal. Cells were counted under 60 X magnification by a person blinded to the treatments.

Distances from the edge of the fiber track to various points in the counted volume were determined by constructing a figure (1B) depicting the administering and counting areas, measuring between the points of interest and converting to tissue distances by applying the appropriate scale factor. This gave a distance from the edge of the fiber track to the middle of the near dorsal edge of the first section counted (section 11) of 130 μm . Similarly the distance to the center of the overall volume counted was 175 μm (Figure 1), and the distances to the ends of the far bottom edge of the volume counted, the most remote points in that volume, was 240 μm .

Administration of 4 mM glutamate through the fiber gives a glutamate concentration of 500 μM i.e., that produced by spinal cord injury, at 100 μm from the fiber (Liu et al., 1999; Xu et al., 2004). Using an observed 6-fold rate of decrease of the concentration with a doubling of distance (McAdoo et al., 1999) gives a 130 μM glutamate concentration at the center of the counted area when 4 mM glutamate was administered and 325 μM when 10 mM glutamate was administered. Corresponding concentrations of 42 μM and 100 μM were estimated at 240 μm , the maximum distance from the fiber track at which cells were counted. Thus the glutamate concentrations in the areas counted were on average about as close as possible to those produced by injury when different concentrations were administered in different experiments. (The counts were made in the same location relative to the fiber track in all sections counted except those giving Figures 5 and 11 were obtained 3, 6, and 9 mm rostral to the fiber track.) A 500 μM glutamate concentration, i.e., the concentration released upon injury, was estimated to occur at 150 μm from the fiber wall when 10 mM glutamate was administered, nearer than the center of the counted volume, 175 μm from the fiber wall. Thus, administering 10 mM glutamate inside the dialysis fiber gave the level of glutamate released by SCI within the tissue counted. The higher concentration of glutamate was administered to obtain dose-response information, and in the case of neurons, to obtain more robust results than anticipated for treatment with 4 mM glutamate.

An antibody to CC-1 stains astrocytes under some circumstances (McTigue et al., 2001), but not under our conditions (Xu et al., 2004). We further tested the specificity of the antibody to CC-1 to oligodendrocytes by looking for doubly labeled neurons in tissue stained with both a polyclonal antibody against Neurofilament H and a monoclonal antibody against CC-1. No double labeling of neurons was observed, establishing that CC-1 did not stain either neurons or astrocytes in our experiments.

Tissue and cell shrinkage

Tissue shrinkage or swelling can affect the cell densities obtained by counting cells. To rigorously eliminate this problem requires the use of stereological counting procedures (Coggeshall and Lekan, 1996). However, applying stereology would not be straightforward in present experiments, as there is no sharp boundary to the zone of glutamate administration and because of the steeply decreasing concentration of administered glutamate with distance from the administering fiber (McAdoo et al., 1999; see above). To establish whether changes in tissue volume influenced our results, we previously determined cross sectional areas of the spinal cord 24 h after exposure to ACSF versus exposure to 10 mM glutamate (Xu et al., 2004). Administration of 10 mM glutamate through the fiber gave cross sectional areas of the cord that were perhaps slightly (15%) although not significantly smaller ($p = 0.12$) than those produced by exposure to ACSF. If glutamate did cause slight tissue shrinkage, that would increase the cell density, an effect opposite to a possible decrease. Thus changes in tissue volume in response to glutamate exposure did not contribute to our observed decreases in cell densities in response to glutamate exposure. Changes in the sizes of cell bodies following exposure to glutamate would also affect cell counts. In previous work, we found that the sizes of cell bodies did not change in response to the treatments and subsequent processing employed here (Xu et al., 2004), so changes in diameters of cell bodies should not have affected our results. Due to the neglect of stereological issues, our counts do not give absolute densities, but the relative densities they produced should be accurate.

Measures of apoptosis

Sections were TUNEL stained with a kit from Trevigen (Gaithersburg, MD) to explore whether glutamate induced DNA fragmentation in neurons and glia. Animals were exposed to ACSF or glutamate as in other treatments, allowed to wake up, re-anesthetized after 24 h, and transcardially perfused with 800 ml of 4% formalin. The tissue containing the site of

administration was put in 4% paraformaldehyde overnight and then transferred to 70% ethanol. The tissue was then embedded in paraffin, cut into 10 μ m sections and deparaffinized by two 5 min immersions in xylenes. Sections were next dehydrated in a graded series of ethanol concentrations (100% – 70%) and then placed in PBS for 10 min at room temperature, covered with 50 μ l of Proteinase K solution (1:200) for 20 min at 37 °C, and washed in deionized water. The slides were immersed in quenching solution for 5 min, washed in PBS, immersed in 1X TdT labeling buffer, covered with 50 μ l of labeling reaction mixture and incubated at 37 °C for 1 h in a humidity chamber. The sections were then placed in a 1X TdT stop buffer for 5 min, washed again with PBS, incubated for 10 min in Strep HRP solution and washed with PBS. Finally, sections were treated with Blue counterstain and washed in succession in deionized water, ammonia water, tap water, 70–100% ethanol and then xylenes. The sections were mounted on slides with mounting media and coverslipped.

We assayed apoptotic cell death with a specific ELISA kit (Cell Death Detection ELISA Kit, Roche #1 544–675) for the DNA in mono and oligonucleosomes released into the cytoplasm by cleavage of the DNA between histones during apoptosis. After perfusion, spinal segment T10 was taken out and frozen on dry ice. Then the tissue was stored at –70 °C until extraction. We followed the protocol provided by the manufacturer through the whole process. 100 μ l of extraction buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF and protease inhibitor cocktail) was added to each tissue; the tissue was sonicated and then centrifuged at 8000 rpm for 15 min at 4°C. Supernatants were collected and protein concentrations were measured in aliquots with a Bio-Rad protein assay (#500–0006); 100 μ l of sample solution was pipetted into wells on a microplate coated with anti-histone solution and the samples were processed according to the manufacturer's instructions. Absorbance was then read at 405 nm, and the difference between those values was plotted versus time.

Photomicrographs of tissue showing sites of ACSF and glutamate administration by microdialysis are shown elsewhere (Xu et al., 2005).

Treatment Groups

The treatment groups were normal animals (animals that received no surgery), animals exposed to only ACSF administered through a dialysis fiber over the time that other animals so received glutamate solutions, animals exposed to 4 mM glutamate in the fluid inside a microdialysis fiber inserted through the cord, and animals similarly receiving 10 mM glutamate (N = 3 for groups assessed for activated caspase-3 except N = 4 in 7 day experiments; N = 6 for all other groups). Unless otherwise specified, statistical significance was assessed first by two-way ANOVA of results of treatments to evaluate whether there were significant differences for (group X time) among treatments, and if so, followed by pairwise comparisons with a Bonferroni t-test. The SigmaStat 2.03 program package (Systat Software, Inc., Point Richmond CA) was used. All results are reported as $\pm$ standard deviation.

All experiments were approved by the Animal Care and Use Committee of the University of Texas Medical Branch and performed in accord with the NIH Guide for the Care and Use of Laboratory Animals.

RESULTS

Oligodendrocytes

Densities (numbers of cells per 0.011 mm²) of normal oligodendrocytes are given in Figure 2 for the treatment groups at times from 6 h to 28 d after the end of exposure to glutamate. At each sampling time, the densities of surviving cells decreased in the order normal > ACSF

exposed > 4 mM glutamate exposed > 10 mM glutamate exposed ($p < 0.001$ for all of these pairwise comparisons of concentrations, except $p = 0.002$ for 4 mM glutamate versus 10 mM glutamate at 72 h). The reductions in oligodendrocyte densities produced by insertion/ACSF administration show that this procedure by itself caused appreciable damage, as has been reported (Xu et al., 2004). Since 10 mM glutamate reduced the density of oligodendrocytes significantly more than did 4 mM glutamate at all times at which exposure to both was examined, the toxicity of glutamate to oligodendrocytes is dose dependent.

The statistical significances of the differences between oligodendrocyte losses at each pair of adjacent time points in response to each treatment are given in Table 1. Results of comparisons between adjacent time points are given for each treatment to indicate whether cell density changed significantly between those points. The later time point in each comparison was always significantly lower or not different from the preceding one (Figure 2). Henceforth we usually take the effects of glutamate on cell numbers at each time to be the mean cell density following exposure to ACSF (control) minus the corresponding mean following treatment with glutamate.

Significant decreases in the densities of oligodendrocytes exposed to glutamate relative to their densities in tissue exposed only to ACSF (Figure 2) demonstrate that glutamate administration caused considerable loss of oligodendrocytes, as reported previously at 24 h post-administration (Xu et al., 2004). The densities of oligodendrocytes decreased substantially in the first 6 h after exposure to ACSF (Figure 2), decreased slightly further by 72 h and remained essentially unchanged thereafter (Figure 2 and Table 1). During the first 72 h following exposure to 4 mM glutamate inside the fiber, the densities of oligodendrocytes declined substantially and then they remained unchanged for at least 25 days (Figure 2); i.e., there was little oligodendrocyte death beyond 72 h after treatment with 4 mM glutamate. Administration of 10 mM glutamate produced a continuous, significant decline (except from 24 h to 72 h) in oligodendrocyte densities. Subtraction of the mean number of oligodendrocytes present following treatment with 10 mM glutamate from the mean number remaining at the same time following ACSF treatment (data in Figure 2) gave the following average declines in oligodendrocyte density (cells per 0.011 mm^2) in response to 10 mM glutamate: 8 oligodendrocytes at 6 h, 14 at 24 h, 11 at 3 days, 15 at 7 days and 17 cells by 28 days. Thus 47% of the overall reduction in oligodendrocyte density in response to 10 mM glutamate occurred in the first 6 h after exposure, and 82% of the overall loss occurred in the first 24 h.

At 1 h (not shown) and 6 h after ACSF administration, there was staining for activated caspase-3, some in bodies connected to or in contact with CC-1 stained processes (Figure 3A). Superimposed staining for caspase-3 and CC-1 did not produce yellow staining, so these bodies may be parts of separate cells. At 6 h after administration of 10 mM glutamate (Figure 3B), many oligodendrocytes stained positively for activated caspase-3, as demonstrated by yellow nuclei in superimposed images. In nearly all of the oligodendrocytes so stained, cytoplasmic CC-1 staining substantially surrounded caspase-3 immunoreactivity, differing somewhat from the staining following ACSF administration. Caspase-3 staining was clearly associated with CC-1 staining, i.e., oligodendrocytes, given the specificity of CC-1 immunoreactivity for oligodendrocytes. The nuclei of oligodendrocytes (CC-1 labeled cells) but not the associated cytoplasm stained intensely for activated caspase-3 following exposure to 10 mM glutamate at all times and distances examined (Figures 3 and 4).

At 7 days post insult, we counted total and caspase-3-labeled oligodendrocytes at 3, 6, and 9 mm from the fiber track in ACSF exposed, 10 mM glutamate exposed and in normal tissue (Figure 5). At this time and at all of these distances from the site of glutamate administration, 10 mM glutamate administration activated caspase-3 in almost half of the remaining oligodendrocytes. Two-threefold fewer oligodendrocytes were labeled for caspase-3 in ACSF-

exposed than in glutamate-exposed tissue and still fewer in normal tissue. Comparable numbers of caspase-3 labeled oligodendrocytes were present at 3 and 6 mm, with some apparent decline by 9 mm from the site of administration of 10 mM glutamate.

Analysis for variance (ANOVA) indicated that there were strong treatment, distance, and (treatment $\times$ distance) interaction effects (all $p < 0.001$). We then conducted separate one-way ANOVAs at each distance to compare mean oligodendrocytes counts across treatments, each of which was highly significantly different from the others (all three $P < 0.001$), indicating strong treatment differences at each distance. Pairwise comparisons using the Bonferroni method produced the results in Table 2.

Apoptosis induced by exposure to glutamate was also assessed by an apoptosis-specific ELISA (Figure 6). This demonstrated a peak response at or before 6 h post-treatment, the interval of most cell death, a much diminished response at 24 h and only a slight response at 28 days; that is, apoptosis diminished as cell death declined. Although this assay was not cell specific, the death of many oligodendrocytes soon after exposure to glutamate undoubtedly involved DNA fragmentation and apoptosis, as was demonstrated by superposition of CC-1 and TUNEL immunostaining (Figure 7). At 24 h after exposure to glutamate, TUNEL staining labeled a substantial number of cells in the gray matter (Figure 6A). Glial cells with apoptotic features were identified by their darkly stained nuclei and relatively small amounts of cytoplasm. Cells with prominent nucleoli and substantial amounts of cytoplasm characteristic of neurons were also TUNEL stained (Figure 7).

Neurons

At all times following administration of 10 mM glutamate in the microdialysis fiber, the densities of neurons were significantly lower than those present after no treatment or exposure to ACSF (Figure 8; $p < 0.001$ for the (group $\times$ time) interaction according to a two-way ANOVA). Subsequent pairwise comparisons with a Bonferroni t-test demonstrated significant differences at each time (Table 2). This reproduces destruction by glutamate of spinal cord neurons observed previously at 24 h after exposure to glutamate (Liu et al., 1991, 1999). As for oligodendrocytes, reduction of neuron densities following exposure to glutamate was due to the combined effects of fiber insertion/ACSF and glutamate exposure, as the losses due to the former as well as those due to the latter were significant. Neuron density in untreated tissue did not vary significantly (20–22 neurons per 0.011 mm^2) from 0 to 28 days (Figure 8). Subtraction of the mean number of oligodendrocytes present following treatment with 10 mM glutamate from the mean number remaining at the same time point following ACSF treatment (data in Figure 2) gave the following average declines in the densities of neurons in response to 10 mM glutamate: 4 in the first 6 h, 6 by 24 h, 8 at day 3, 8 at day 9, and 8 by day 28. There was not significant neuron loss between 72 h and 28 d after exposure to 10 mM glutamate (Table 3). The density of neurons remaining at each time point after exposure to glutamate was significantly lower than the density of neurons surviving treatment with ACSF at the same time ($p < 0.001$ for all; two way ANOVA followed by the Bonferroni t-test).

There was no sign of activated caspase-3 in normal neurons, but it was present in some ACSF-exposed neurons at 6 h post-exposure (Figure 9). At 1 h (not shown) and 6 h after administration of 10 mM glutamate, caspase-3 was activated in most neurons and significantly more neurons expressed caspase-3 than at the same times after ACSF administration. As with oligodendrocytes, caspase-3 activated by 10 mM glutamate administration was strongly localized in the nuclei of neurons at 1 h (Figure 10), 6 h (Figure 9) and 7 days.

To characterize the spread of the effects of glutamate, we assessed caspase-3 activation in neurons 3, 6 and 9 mm rostral to the site of administration of 10 mM glutamate at 7 days after its administration (Figure 11). Activated caspase-3 was present in the nuclei of motor neurons

at all three distances from the site of administration at 7 days after administration of 10 mM glutamate. Fewer, but still a substantial number of neurons at these same distances were labeled for activated caspase-3 following administration of ACSF. Labeling of neurons at 9 mm from the fiber track in response to ACSF was much less intense in number and density than labeling closer to the fiber track. Neurons from untreated animals did not stain for caspase-3. The F-test indicated significant effects of treatment ($p < 0.001$). Subsequent one way analysis of variance with Bonferroni corrections gave the following p values: Glutamate versus ACSF < 0.01 , ACSF versus normal < 0.001 , ACSF versus normal 0.029.

DISCUSSION

Relationship of cell loss to locomotor dysfunction

This work characterizes the adverse effects of elevated glutamate on spinal cord oligodendrocytes and neurons. From glutamate administration until 28 days later, both numerical scores of locomotor function (Xu et al., 2005) and the densities of surviving oligodendrocytes (present work) at each time point were almost all in the order: normal $>$ ACSF $>$ 4 mM glutamate $>$ 10 mM glutamate. However, although loss of motor neurons can impair hindlimb function (Magnuson et al., 1999, 2005; Collazos-Castro et al., 2005), it is unlikely that it did so in present experiments because the hindlimb motoneurons are in segment L2 (Magnuson et al., 2005), far enough (24 mm) caudal to the site of glutamate administration (T10) not to be exposed to administered glutamate. Defective axonal conduction due to the loss of oligodendrocytes is probably a major cause of the hindlimb locomotor deficits produced by administering glutamate into the cord at segment T10 (Xu et al., 2005), as defective conduction in the long axons appears to underlie the impairment of hindlimb functions caused by thoracic insult (Blight, 1983; Teng and Wrathall, 1997; Cao et al., 2005). The functional recovery that occurs over the first few weeks following SCI at spinal segment T10 (Xu et al., 2005) undoubtedly reflects recovery of the transmission of electrical signals. Given that we observed no increase of oligodendrocyte numbers during this time, if remyelination was important to recovery, it was accomplished by surviving rather than newly generated oligodendrocytes. Axonal sprouting to make new connections is a likely source of functional recovery (Goldberger and Murray, 1977; Christensen and Hulsebosch, 1997). Since neuron numbers around the site of insult decrease slowly or remain constant at longer times following exposure to glutamate, functional recovery is not due to the generation of additional neurons.

According to assessment by the BBB test (Xu et al., 2005), administering the levels of glutamate released following SCI caused hindlimb impairments which resembled those generated by contusion injury (Basso et al., 1995; Scheff et al., 2003). Locomotion was most reduced at 1–2 days after 10 mM glutamate administration and gradually improved over the next 4 weeks (Xu et al., 2005). This time course is similar to that of the loss of function and its recovery following contusion SCI (Basso et al., 1995; Kloos et al., 2005) and that following unilateral dorsal rhizotomy (L1-S1) (Goldberger and Murray, 1988). In experiments parallel to the present ones, ACSF administration caused impairments of locomotion that lasted for 7 to 28 days and rearing deficits to beyond 28 days after the insult. Glutamate-induced deficits in movements were significantly greater than those caused by ACSF (Xu et al., 2005).

In addition to having commonalities, responses to a contusion insult and glutamate administration undoubtedly also differ because of differences in mechanical damage, effects on the circulation, hypoxia and in other factors between the two types of injury. Nonetheless, the similarity of glutamate-induced functional impairments following recovery (Xu et al., 2005) to those caused by contusion injury (Basso et al., 2005) and dorsal rhizotomy (Goldberger and Murray, 1988) suggest that varied insults to the spinal cord ultimately cause damage through related mechanisms, probably in each case causing cell damage and death. Since the levels of glutamate administered approximate the tissue levels reached upon SCI and

mechanical damage is generated by both microdialysis fiber insertion and contusion models of SCI, the results presented here should be relevant to post-SCI mechanisms of cell death and functional impairment.

Glutamate-induced loss of oligodendrocytes

Present administration of glutamate into the spinal cord at around the levels reached following SCI destroyed many oligodendrocytes. Around 136 kM (the center of the counted zone, see Methods) from the site of administration, the densities of oligodendrocytes decreased substantially by 6 h and then more gradually until 24 h after administration of 4 mM and 10 mM glutamate by microdialysis (Figure 2). These densities changed little beyond 24 h after administration of 4 mM glutamate, but they did decline further following exposure to 10 mM glutamate. Equating the rate of cell loss to rate of cell death assumes that there was no replacement of oligodendrocytes over this time; however, replacement of oligodendrocytes could influence our results, as it can follow contusion injury (McTigue et al., 2001).

The substantial loss of oligodendrocytes in response to microdialysis fiber insertion/ACSF perfusion suggests that cells may be sensitized by this procedure such that their loss is enhanced in response to exposure to glutamate, causing cell losses attributed to the toxicity of glutamate to exceed the loss that would be produced by glutamate in the absence of other factors. This is quite possible, as mechanical stretch increases neuron death in response to exposure to low levels of NMDA (Arundine et al., 2004). However, much more damage is probably caused by a contusion SCI than by insertion of a microdialysis fiber, as microdialysis fiber insertion alone causes relatively little apparent histological damage away from the narrow fiber track (Xu et al., 2005), whereas the damage to tissue done by even a “mild to moderate” impact is quite extensive (Basso et al., 1995). Therefore sensitization by injury in combination with exposure to released glutamate is probably more substantial in a contusion SCI model than it is following administration of glutamate by microdialysis. Thus sensitization to glutamate by insertion of a microdialysis probe, if it occurs, likely models the effect of glutamate on damaged cells that follows SCI. This is worth pursuing.

Present results demonstrate that the brief elevation of glutamate that follows SCI (McAdoo et al., 1999) causes caspase-3 activation, DNA fragmentation and cell death. This is consistent with previous reports that many oligodendrocytes die by apoptosis following SCI (Crowe et al., 1997; Shuman et al., 1997; Beattie et al., 1998, 2000; Young et al., 1998; Wada et al., 1999; Grossman et al., 2001; McTigue et al., 2001; Casha et al., 2001; Park et al., 2003, 2004). In our ELISA measures of apoptosis, the level of apoptosis declined to very low levels by 28 days post-treatment; a decline over the same time frame as the decline in the rate of cell loss. In previous investigations, contusion SCI produced an early and a later peak of apoptotic oligodendrocytes (Liu et al., 1997; Yong et al., 1998; McEwen and Springer, 2005), and apoptosis of oligodendrocytes spreads rostrally and caudally from the epicenter for at least three weeks post injury (Crowe et al. 1997; Shuman et al., 1997). McTigue et al. (2001) reported a decrease in oligodendrocyte numbers at 7 days post injury followed by an increase at 14 days due to the generation of new oligodendrocytes, and then a final decrease. A rise in activated caspase-3 during the first day after injury followed by a decrease for several days and then another rise in caspase-3 labeled oligodendrocytes following SCI was observed recently (McEwen and Springer, 2005). McEwen and Springer also reported that increases and decreases in cells containing caspase-3 and in CC-1 labeled cells occur in parallel following contusion SCI. Our observation of staining of oligodendrocytes and neurons for caspase-3 out to 7 days, the longest time of assessment for caspase-3 following exposure to glutamate, is consistent with earlier reports of caspase-3 expression in oligodendrocytes for 2–3 weeks post SCI (Crowe et al., 1997; McTigue et al., 2001). However, we did not detect any peaks or dips in oligodendrocyte numbers over 6 h to 28 d in our experiments (Figure 2). Thus

oligodendrocytes expressing caspase-3 probably persisted rather than dying and being replaced following exposure to glutamate in present work.

Many cells disappeared quickly following exposure to glutamate. Some, if not most, of the oligodendrocytes that died by 6 h after exposure to glutamate very likely disappeared by apoptotic mechanisms, given that: 1) Staining of oligodendrocytes for activated caspase-3 was present by 1 h and prominent by 6 h post-exposure. 2) TUNEL and CC-1 staining were co-localized in some cells (Figure 7), 3) there was a strong peak in apoptosis at 6 h after treatment with glutamate according to an apoptosis-specific ELISA (Figure 6), and many TUNEL-stained glia were present at 24 h after exposure to 10 mM glutamate. However, many oligodendrocytes with apoptotic features were still present at seven days. Our evidence that glutamate exposure caused considerable apoptosis in both neurons and oligodendrocytes is strong, but given that DNA fragmentation can also accompany necrosis (Gwag et al., 1997; Portera-Cailliau et al., 1997; Sastry and Rao, 2000), necrosis may have contributed to apparent apoptosis.

Glutamate-induced loss of neurons

As with oligodendrocytes, the rate of neuron loss maximized by 6 h after exposure to 10 mM glutamate and became negligible beyond 3 days. However, caspase-3 labeled neurons were abundant at 7 days after administration of 10 mM glutamate, and neuron numbers did not change significantly from 3 to 28 days after glutamate exposure (Figure 8). Therefore many neurons containing activated caspase-3 were not dying or were dying very slowly. Several groups have reported the presence of apoptotic neurons post-SCI (Liu et al., 1997; Beattie et al., 1998; Yong et al., 1998; Wada et al., 1999; Qiu et al., 2001), although others have not found them (Casha et al., 2001; Grossman et al., 2001), a puzzling inconsistency. In vitro experiments suggest that excitotoxicity kills neurons primarily by necrosis (Gwag et al., 1997; Zipfel et al., 2000). However, the elevated expression of caspase-3 in neurons might be additional evidence that substantial numbers of the cells that disappeared in response to exposure to glutamate died by apoptosis, given that caspase-3 is thought to be a mediator of apoptosis.

As with oligodendrocytes, at 7 days after glutamate administration, neurons containing activated caspase-3 were present to at least 9 mm from the site of administration (Figure 11). However, microdialysis administration does not elevate glutamate beyond 3 mm from the dialysis fiber (McAdoo et al., 1999), suggesting that cell damage spreads by intermediaries other than to diffusing glutamate. Another reason for thinking that the intermediary is not diffusing glutamate is that released glutamate does not elicit substantial further glutamate release following SCI (McAdoo et al., 2005). Microglia may contribute to this spread, as they appear to be involved in the spread of apoptosis of oligodendrocytes following contusion SCI (Shuman et al., 1997).

Caspase-3 activation

Substantial evidence demonstrates that activation of caspase-3 contributes to the development of deficiencies caused by SCI (Springer et al., 1999; Li and Stys, 2000). Blocking caspase-3 activation following SCI promotes survival of oligodendrocytes, implicating caspase-3 in the post-SCI losses of those cells (Nottingham et al., 2002). Caspase-3 was activated in many oligodendrocytes and neurons after exposure to glutamate. This correlates with detection of such activity in extracts of injured spinal cords after spinal cord injury (Springer et al., 1999).

The very slow decrease in densities of oligodendrocytes during the presence of many caspase-3 expressing cells post-glutamate exposure demonstrates that, as has been reported (Zeuner et al., 1999), activation of caspase-3 is not followed by rapid cell death in all cells. This suggests functions of caspase-3 other than mediating cell death in response to glutamate and hence to

SCI. At 7 days after glutamate administration, many caspase-3-containing oligodendrocytes and neurons were present out to at least 9 mm from the site of administration (Figures 5 and 11). However, administration of glutamate by microdialysis does not elevate glutamate beyond 3 mm from the dialysis fiber (McAdoo et al., 1999), suggesting that cell damage spreads to greater distances by intermediate processes rather than simple exposure to diffusing glutamate. Oligodendrocytes also stained for caspase-3 until at least d 7 following contusion SCI, although a non-staining interval of several days has been reported following SCI (McEwen and Springer, 2005). Caspase-3 can also appear and disappear in neurons in less than 96 h in response to the administration of the glutamate agonist kainate (Nottingham and Springer, 2003). There is no sign of such variations in our data, although such might have occurred between the times of sampling.

It is unlikely that apoptosing cells survive and recover their function (Bursch et al., 1990), but it is quite possible that many of the caspase-3-labeled cells observed in this work were not dying, given the presence of many caspase-3 labeled neurons at a time when numbers of oligodendrocytes and neurons were declining at most very slowly or not at all. Alternatively, caspase-3 containing cells may function temporarily until other mechanisms, such as sprouting by surviving neurons to make new contacts, can produce lasting recovery of function (Goldberger and Murray, 1988).

Examination of published images revealed instances of cell nuclei stained for caspase-3 (McEwen and Springer, 2005), localization to cytoplasm following contusion SCI (Springer et al., 2004), or a substantial presence in both regions following a partial transection (Springer et al., 2004). This variability in the cellular localization of activated caspase-3 suggests that in SCI different pathways to activation of caspase-3 are initiated by different insults. Supporting this, the role of caspase-3 in apoptosis can be quite context dependent (Woo et al., 2006). Activated caspase-3 has substrates in both the cytoplasm and in the nucleus, substrates whose breakdown induces apoptotic changes in cytoplasmic and nuclear morphology (Zheng et al., 1998; Eldadah and Faden, 2000). Nuclear accumulation of activated caspase-3 in cultured HepG2 cells involves cleavage of procaspase-3 in the cytoplasm and transport of the products to the nucleus during apoptosis (Kamada et al., 2005). Glutamate may initiate analogous processes in the spinal cord, as activated caspase-3 is required for DNA fragmentation during apoptosis (Jänicke et al., 1998). Nuclear localization of activated caspase-3 deserves further attention in CNS trauma, as it may be important in secondary damage during SCI or even perhaps even in counteracting it.

SUMMARY

We demonstrated that exposure to glutamate produces substantial losses of oligodendrocytes and neurons accompanied by apoptotic features: activation of caspase-3 and TUNEL reaction. Most glutamate release occurs within about 1 h after SCI, and cell loss and DNA cleavage are most prominent around 6 h post-glutamate treatment in present experiments. Caspase-3 expression in oligodendrocytes and neurons persists for at least one week after exposure to glutamate. Some recovery of function occurs over several weeks post-SCI during which there are slight decreases in the densities of oligodendrocytes and neurons. Caspase-3 is strongly and persistently localized to cell nuclei following administration of glutamate and ACSF; this surprising observation suggests that the nucleus is an important site of action of caspase-3, not necessarily adverse, during excitotoxicity.

Acknowledgements

We thank Debbie Pavlu for assistance with preparation of the manuscript, James Grady for assistance with statistics, Joe Springer for sharing data, Richard Coggeshall for advice regarding histochemistry, and the reviewers for useful

suggestions. This work was supported by grants from NIH (NS39161 and NS11255) and Mission Connect of the TIRR Foundation.

Abbreviations

ACSF	artificial cerebrospinal fluid
ANOVA	analysis of variance
DTT	dithiothreitol
EDTA	ethylene diamine tetraacetic acid
NGS	normal goat serum
PBS	phosphate buffered saline
SCI	spinal cord injury

References

- Basso DM, Beattie MS, Bresnahan JC. A sensitive and reliable locomotor rating scale for open field testing in rats. *J Neurotrauma* 1995;12:1–21. [PubMed: 7783230]
- Beattie MS, Shuman SL, Bresnahan JC. Apoptosis and spinal cord injury. *Neuroscientist* 1998;4:163–171.
- Bhat RV, Axt KJ, Fosnaugh JS, Smith KJ, Johnson KA, Hill DE, Kinzler KW, Baraban JM. Expression of the APC tumor suppressor protein in oligodendroglia. *Glia* 1996;7:169–174. [PubMed: 8776583]
- Cao Q, Zhang YP, Iannotti C, DeVries WH, Xu X-M, Shields CB, Whittemore SR. Functional and electrophysiological changes after graded traumatic spinal cord injury in adult rat. *Exp Neurol* 2005;191:S3–S16. [PubMed: 15629760]
- Casha S, Yu WR, Fehlings MG. Oligodendroglial apoptosis occurs along degenerating axons and is associated with fas and p75 expression following spinal cord injury in the rat. *Neuroscience* 2001;103:203–218. [PubMed: 11311801]
- Christensen MD, Hulsebosch CE. Spinal cord injury and anti-NGF treatment results in changes in CGRP density and distribution in the dorsal horn of the rat. *Exp Neurol* 1997;147:463–475. [PubMed: 9344570]
- Coggeshall RE, Lekan HA. Methods for determining numbers of cells and synapses: a case for more uniform standards of review. *J Comp Neurol* 1996;364:6–15. [PubMed: 8789272]
- Crowe MJ, Bresnahan JC, Shuman SL, Masters JN, Beattie MS. Apoptosis and delayed degeneration after spinal cord injury in rats and monkeys. *Nature Med* 1997;3:73–76. [PubMed: 8986744]
- Eldadah BA, Faden AI. Caspase pathways, neuronal apoptosis and CNS injury. *J Neurotrauma* 2000;10:811–829. [PubMed: 11063050]
- Faden AI, Demediuk P, Panter SS, Vink R. The role of amino acids and NMDA receptors in traumatic brain injury. *Science* 1989;244:798–800. [PubMed: 2567056]
- Faden AI, Lemke M, Simon RP, Noble LJ. N-methyl-D-aspartate antagonist MK801 improves outcome following traumatic spinal cord injury in rats: Behavioral, anatomic, and neurochemical studies. *J Neurotrauma* 1988;5:33–45. [PubMed: 3057216]
- Faden AI, Simon RP. A potential role for excitotoxins in the pathophysiology of spinal cord injury. *Ann Neurol* 1988;23:623–626. [PubMed: 2841902]

- Grossman SD, Rosenberg LJ, Wrathall JR. Temporal-spatial pattern of acute neuronal and glial loss after spinal cord contusion. *Exp Neurol* 2001;168:273–282. [PubMed: 11259115]
- Gwag BJ, Koh JY, Demaro JA, Ying HS, Jacquin M, Choi DW. Slowly triggered excitotoxicity occurs by necrosis in cortical cultures. *Neuroscience* 1997;77:393–401. [PubMed: 9472399]
- Li S, Stys PK. Mechanisms of ionotropic glutamate receptor-mediated excitotoxicity in isolated spinal cord white matter. *J Neurosci* 2000;20:1190–1198. [PubMed: 10648723]
- Liu D, Thangnipon W, McAdoo DJ. Excitatory amino acids rise to toxic levels upon impact injury to the rat spinal cord. *Brain Res* 1991;547:344–348. [PubMed: 1884213]
- Liu D, Xu G-Y, Pan E, McAdoo DJ. Neurotoxicity of glutamate at the concentration released upon spinal cord injury. *Neuroscience* 1999;93:1383–1389. [PubMed: 10501463]
- Liu XZ, Xu XM, Hu R, Du C, Zhang SX, McDonald JW, Dong HX, Wu YJ, Fan GS, Jacquin MF, Hsu CY, Choi DW. Neuronal and glial apoptosis after traumatic spinal cord injury. *J Neurosci* 1997;17:5395–5406. [PubMed: 9204923]
- Magnuson DSK, Trinder TC, Zhang YP, Burke D, Morassutti DJ, Shields CB. Comparing deficits following excitotoxic and contusion injuries in the thoracic and lumbar spinal cord of the adult rat. *Exp Neurol* 1999;156:191–204. [PubMed: 10192790]
- McAdoo DJ, Hughes MG, Nie L, Shah B, Clifton C, Fullwood S, Hulsebosch CE. The effect of glutamate receptor blockers on glutamate release following spinal cord injury. Lack of evidence for an ongoing feedback cascade of damage → glutamate release → damage → glutamate release → etc. *Brain Res* 2005;1038:92–99. [PubMed: 15748877]
- McAdoo DJ, Xu G-Y, Robak G, Hughes MG. Changes in amino acid concentrations over time and space around an impact injury and their diffusion through the rat spinal cord. *Exp Neurol* 1999;159:538–544. [PubMed: 10506525]
- McTigue DM, Wei P, Stokes BT. Proliferation of NG2-positive cells and altered oligodendrocyte numbers in the contused rat spinal cord. *J Neurosci* 2001;21:3392–3400. [PubMed: 11331369]
- Noble LJ, Wrathall JR. Correlative analyses of lesion development and functional status after graded spinal cord contusive injuries in the rat. *Exp Neurol* 1989;103:34–40. [PubMed: 2912748]
- Panter SS, Yum SW, Faden AI. Alteration in extracellular amino acids after traumatic spinal cord injury. *Ann Neurol* 1990;27:96–99. [PubMed: 2301932]
- Park E, Liu Y, Fehlings MG. Changes in glial cell white matter AMPA receptor expression after spinal cord injury and relationship to apoptotic cell death. *Exp Neurol* 2003;182:35–48. [PubMed: 12821375]
- Park E, Velumian AA, Fehlings MG. The role of excitotoxicity in secondary mechanisms of spinal cord injury: a review with an emphasis on the implications for white matter degeneration. *J Neurotrauma* 2004;21:754–774. [PubMed: 15253803]
- Qiu J, Nesic O, Ye Z, Rea H, Westlund KN, Xu G-Y, McAdoo DJ, Hulsebosch CE, Perez-Polo JR. Bcl-x_L expression after contusion to the rat spinal cord. *J Neurotrauma* 2001;11:1267–1278.
- Rosenberg LJ, Teng YD, Wrathall JR. 2,3-Dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline reduces glial loss and acute white matter pathology after experimental spinal cord contusion. *J Neurosci* 1999;19:464–475. [PubMed: 9870974]
- Sastry PS, Rao KS. Apoptosis and the nervous system. *J Neurochem* 2000;74:1–20. [PubMed: 10617101]
- Shuman SL, Bresnahan JC, Beattie MS. Apoptosis of microglia and oligodendrocytes after spinal cord contusion in rats. *J Neurosci Res* 1997;50:798–808. [PubMed: 9418967]
- Springer JE, Azbill RD, Knapp PE. Activation of the caspase-3 apoptotic cascade in traumatic spinal cord injury. *Nature Med* 1999;5:943–946. [PubMed: 10426320]
- Teng YD, Wrathall JR. Local blockade of sodium channels by tetrodotoxin ameliorates tissue loss and long-term functional deficits resulting from experimental spinal cord injury. *J Neurosci* 1997;17:4359–4366. [PubMed: 9151752]
- Wada S, Yone K, Ishidou Y, Nagamine T, Nakahara S, Niiyama T, Sakou T. Apoptosis following spinal cord injury in rats and preventative effect of *N*-methyl-D-aspartate receptor antagonist. *J Neurosurg* 1999;91:98–104. [PubMed: 10419375]
- Waxman SG. Demyelination in spinal cord injury. *J Neurol Sci* 1989;91:1–14. [PubMed: 2664092]

- Xu G-Y, Hughes MG, Ye Z, Hulsebosch CE, McAdoo DJ. Concentrations of glutamate released following spinal cord injury kill oligodendrocytes in the spinal cord. *Exp Neurol* 2004;187:329–336. [PubMed: 15144859]
- Xu G-Y, Hughes MG, Zhang L, Cain L, McAdoo DJ. Administration of glutamate into the spinal cord at extracellular concentrations reached post-injury causes functional impairments. *Neurosci Lett* 2005;384:271–276. [PubMed: 15925447]
- Yong C, Arnold PM, Zoubine MN, Citron BA, Watanabe I, Berman NEJ, Festoff BW. Apoptosis in cellular compartments of rat spinal cord after severe contusion injury. *J Neurotrauma* 1998;15:459–472. [PubMed: 9674550]
- Zipfel GJ, Babcock DJ, Lee J-M, Choi DW. Neuronal apoptosis after CNS injury: the roles of glutamate and calcium. *J Neurotrauma* 2000;17:857–869. [PubMed: 11063053]

Figure 1.

Diagram showing: A The lateral and dorsoventral relationships between the counted areas (small squares) and the fiber track (dashed lines drawn across the section). The fiber was inserted directly below dorsal root T10 and passed through the cord near its dorsal-ventral center. The counted areas extend ventrally from the ventral edge of the fiber track in the white and gray matter. The counted areas were 105 μm X 105 μm squares. B. The dorsoventral and rostral-caudal relationships between the sections counted and the fiber track. The vertical dashed line indicates the relationship between the fiber track and the beginning of the 10 adjacent sections used to locate sections for counting. For calibration, the sum of the thicknesses of these 10 sections equals 100 μm . The numbers across the bottom of the figure

label 10 km sections starting at the edge of the fiber track. Cells were counted in sections 11–15. Other numbers on the figure are the minimum distances (in km) from a counted section to the fiber track, the distance of the farthest counted section from the fiber track, the distance from the fiber track to the center of the total volume of tissue in which cells were counted, and the furthest distance of counting from the fiber track. These distances are derived by measurements on the figure - 1 section width on the figure = 10 km in the tissue. The estimated glutamate concentrations in the volume counted were 290 kM to 42 kM for 4 mM glutamate and 725 to 100 kM during administration of 10 mM glutamate (See Methods).

Figure 2.

Time dependency of the losses of oligodendrocytes following exposure to glutamate administered by microdialysis. Glutamate or ACSF was administered through a microdialysis fiber inserted laterally through the spinal cord. Oligodendrocytes were counted in 105 X 105 km areas at distances in the range 100 – 183 km from the track of the microdialysis fiber (See Figure 1) at 6 h to 28 days post-exposure. Note the rapid initial decline followed by a substantially decreasing rate of decline in the densities of oligodendrocytes. Also note that inserting the microdialysis probe and perfusing it with ACSF produced significant loss of cells, as discussed in the text. Each point on the plots is made up of data from animals used only for that point. Statistical evaluation is given in Results. Error bars are $\pm$ s.d.

Figure 3.

The effects of administering 10 mM glutamate on oligodendrocytes at 6 h post-exposure. From left to right, the panels represent visualization of 1) CC-1 labeled oligodendrocytes, 2) activated caspase-3 (arrows) and 3) merger of panels 1 and 2. Areas counted were located as described in Figure 1. A. Effect of ACSF administration on oligodendrocytes at 6 h after exposure. Note that some bodies in A3 containing activated caspase-3 are in contact with, but not surrounded by, green-stained processes. Thus it is not clear whether the caspase-3 stained bodies and green stained processes are parts of the same or separate cells. Caspase-3 is not yet activated by ACSF in most of the oligodendrocytes. B. The effect of 10 mM glutamate on oligodendrocytes at 6 h post-administration. Note that a major fraction of the oligodendrocytes expressed caspase-3 (green bodies with red, yellow and/or white centers in B3). Each arrow points to one or two cells that labeled for both caspase-3 and CC-1. Scale bar = 5 μ m.

Figure 4. Immunohistochemistry of oligodendrocytes demonstrating nuclear localization of caspase-3 at 6 h after administration of 10 mM glutamate. A. Stained with CC-1. B. Stained with an antibody to activated caspase-3, and C, Merger of A and B. The orange staining in the centers is nuclear staining for caspase-3. Scale bar = 5 μ m.

Figure 5.

Counts of total oligodendrocytes and oligodendrocytes containing activated caspase-3 as a function of distance (3, 6 and 9 mm from the site of administration). Sections were counted in the same location dorso-ventally and laterally from the site of administration of 10 mM glutamate as in other experiments. Top panel – The clear bar in each pair represents densities of total (caspase-3 stained plus unstained) oligodendrocytes; the associated dark bar represents the densities of caspase-3-containing oligodendrocytes. Nearly half of the oligodendrocytes contained activated caspase-3 at 3 and 6 mm, and fewer at 9 mm from the track of a fiber used to administer 10 mM glutamate. Tissues were taken 7 days after exposure to glutamate. Bottom panel: Numbers of caspase-3 labeled oligodendrocytes generated over distance in control (normal), ACSF treated and 10 mM glutamate exposed animals. Note the increased caspase-3 activation following exposure to glutamate and the apparent decline in the number of oligodendrocytes containing activated caspase-3 with increasing distance from the fiber following glutamate administration.

Figure 6. Results of an ELISA assay to assess overall apoptosis. Maximum overall apoptosis occurred near 6 h after the administration of 10 mM glutamate followed by a decline over time as cell death diminished.

Figure 7.

Upper panel - TUNEL staining of cells in the ventral gray matter indicating DNA cleavage at 24 h after exposure to 10 mM glutamate. The area displayed was 100 μ m from the fiber track located as described in Methods. The cells with smaller, round, more darkly stained nuclei (dark blue arrows) are glial cells and the cells with the larger, prominent nuclei and substantial cytoplasm are neurons (light blue arrows). Scale bar is 10 μ m. Lower panels. Colocalization of TUNEL and CC-1 staining at 1 h after administration of 10 mM glutamate into the spinal cord. A. TUNEL staining (green). B. CC-1 staining (red). C. Merger of A and B showing coincidence of CC-1 and TUNEL staining. These results demonstrate that elevated glutamate quickly produces apoptosis in oligodendrocytes. Scale bars C1 = 5 μ m; C2 = 25 μ m.

Figure 8.

Neuron loss over time following the administration of 10 mM glutamate or ACSF into the spinal cord by microdialysis. Each point is from a different group of animals. Note that some neurons disappeared in response to microdialysis fiber insertion plus ACSF administration, but more were lost in response to glutamate administration. Also note that loss was initially rapid, but the rate slowed considerably with increasing time, that is most loss occurred within about 6 h after fiber insertion and glutamate administration. Sections for counting were taken 100 – 150 μ m rostral from the fiber track (Figure 1) and counted in areas ventral to the level of the track. See Results for statistical evaluations. Error limits are $\pm$ s.d.

Figure 9.

Immunocytochemical characterization at 6 h post-exposure of the effects of administration of 10 mM glutamate by microdialysis on spinal cord neurons. Sections were stained with 1) an antibody to Neu-N to visualize neurons (green), and 2) an antibody to activated caspase-3. 3) Merged image of panels 1 and 2. A. Control tissue from an untreated rat, B. Tissue from an ACSF exposed rat, and C. Tissue from a rat to which 10 mM glutamate was administered through a microdialysis fiber. Arrows point to neurons labeled for activated caspase-3. Scale bar = 10 μ m for all images. Note that more neurons were labeled more intensely after exposure to glutamate than after exposure to ACSF. Sections were taken at locations described in Figure 1.

Figure 10.

Neurons labeled for activated caspase-3 after administration of 10 mM glutamate demonstrating nuclear localization of caspase-3. A. 1). Sections taken at 1 h after exposure to 10 mM glutamate and stained for activated caspase-3. 2) Section stained for Neu-N + caspase-3. B. As in A, except the tissue was taken at 6 h after administration of 10 mM glutamate. Note that activated caspase-3 is essentially confined to the nuclei of the neurons (cells with green cytoplasm). Scale bar = 5 μ m.

Figure 11.

Total and activated caspase-3-containing neurons 7 days after administration of 10 mM glutamate. Top panel. At the time and distances sampled (3 mm, 6 mm and 9 mm rostrally from the site of administration), most of the neurons were labeled for activated caspase-3. Neurons were identified by Neu-N labeling. Bottom panel. Densities of caspase-3 labeled neurons as a function of distance from the fiber track and treatment (normal, ACSF or 10 mM glutamate). The densities of caspase-3 increased in the order normal < ACSF < glutamate at all distances. Barely perceptible caspase-3 labeling was present in some neurons at 9 mm from the fiber track after exposure to ACSF. These were not counted as caspase-labeled cells. The effect of ACSF declined with distance from the fiber track, but that of glutamate did not over the distances sampled, indicating stronger effects of glutamate than of ACSF.

Table 1

Significances (p) of differences between densities of oligodendrocytes at adjacent time points following exposure to glutamate

Treatment	24 h vs. 6 h	72 h vs. 24 h	7d vs 72 h	28 d vs 7 d
Normal	1.000	0.676	1.000	1.000
ACSF	1.000	< 0.001	1.000	1.000
4 mM glu	< 0.001	< 0.032	1.000	1.000
10 mM glu	< 0.001	0.961	0.019	0.052

Effects of treatments on densities of cells were determined by cell counting as described in Methods (n = 6 animals/group). A two factor ANOVA gave a significant (group X time) interaction (p < 0.001). The p values in this table were obtained by following the ANOVA with a Bonferroni t-test to compare adjacent densities.

Table 2

Significance of effects of treatment at each distance sampled on the number of oligodendrocytes expressing caspase-3.

Distance	Comparison		
	Glu vs. ACSF	Glu vs normal	ACSF vs. normal
3 mm	<0.001	< 0.001	0.0049
6 mm	< 0.001	< 0.001	0.1938
9 mm	< 0.001	< 0.001	0.1016

Table 3

Statistical comparison of numbers of surviving neurons over time after exposure to 10 mM glutamate

6 h	24 h	72 h	9 days	28 days
< 0.001	<0.001	< 0.001	1.00	1.00

P values are for comparison of the density of surviving neurons present at each time point to the density at the preceding time point; n = 6 animals per group. There is a clear decline in the density of neurons present in the naïve state to 3 days post SCI, with stabilization of these densities after 3 days