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Stress exacerbates neuron loss and microglia proliferation in a rat model of excitotoxic lower motor neuron injury

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ABSTRACT

All individuals experience stress and hormones (e.g., glucocorticoids/GCs) released during stressful events can affect the structure and function of neurons. These effects of stress are best characterized for brain neurons; however, the mechanisms controlling the expression and binding affinity of glucocorticoid receptors in the spinal cord are different than those in the brain. Accordingly, whether stress exerts unique effects on spinal cord neurons, especially in the context of pathology, is unknown. Using a controlled model of focal excitotoxic lower motor neuron injury in rats, we examined the effects of acute or chronic variable stress on spinal cord motor neuron survival and glial activation. New data indicate that stress exacerbates excitotoxic spinal cord motor neuron loss and associated activation of microglia. In contrast, hypertrophy and hyperplasia of astrocytes and NG2+ glia were unaffected or were modestly suppressed by stress. Although excitotoxic lesions cause significant motor neuron loss and stress exacerbates this pathology, overt functional impairment did not develop in the relevant forelimb up to one week post-lesion. These data indicate that stress is a disease-modifying factor capable of altering neuron and glial responses to pathological challenges in the spinal cord.

1. Introduction

All individuals experience stress. The cellular and molecular consequences of stress, largely mediated by glucocorticoids (GCs) binding to glucocorticoid receptors (GRs), can profoundly affect neuronal structure and function. These effects are best documented for neurons in the hippocampus where stress and GCs affect synaptogenesis and the signal transduction pathways responsible for learning and memory (reviewed in Kim and Diamond, 2002). In the context of brain injury or disease, GCs exacerbate neuroinflammation and neuron loss in experimental models of seizure, stroke or brain inflammation (Dinkel et al., 2003; MacPherson et al., 2005; Munhoz et al., 2006, 2010; Smith-Swintosky et al., 1996; Sorrells et al., 2013, 2009; Stein-Behrens et al., 1992). Comparatively less is known about how spinal cord neurons respond to stress or GCs and because the mechanisms controlling the expression and sensitivity of GRs in the spinal cord are different than in the brain (Moses et al., 1991, 1989; Patacchioli et al., 1998), it is possible that differential effects will occur between these two regions of the central nervous system.

Excitotoxic neuron death and neuroinflammation are pathologic hallmarks of several neurological disorders that uniquely affect the spinal cord (e.g., traumatic spinal cord injury, amyotrophic lateral sclerosis (ALS) and spinal ischemia secondary to trans-abdominal aortic aneurysm repair). To test the hypothesis that stress will place spinal cord neurons at additional risk for injury caused by excitotoxicity or neuroinflammation, a non-traumatic model of focal glutamate microinjection was used. When injected into the spinal cord ventral horn of naïve rats, glutamate kills neurons within discrete spinal laminae without causing bleeding or non-specific trauma. When identical injections are made into rats subjected to acute or chronic variable stress paradigms, neuron cell death was exacerbated and microglia proliferation was increased. Only in stressed rats did neurons die beyond the site of injection; significantly more neurons died several millimeters proximal/distal to the injection site. Despite the exacerbated loss of ventral horn motor neurons, no obvious deficits in gross forelimb function were noted, even several days after the onset of pathology. Together, these data indicate

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that stress is a potent disease-modifying factor that can exacerbate spinal cord neuron pathology.

2. Materials and methods

2.1. Animals

A total of fifty-four adult female Sprague–Dawley rats (Harlan Laboratories; 225–250 g) were used in all experiments. For acute stress experiments (Figs. 1–3), a total of $n = 16$ rats was used. For those experiments, controls consisted of intraspinal PBS (vehicle) injections into stressed or no-stress rats ($n = 4$/group). Since no differences were observed between groups, the groups were combined ($n = 8$/PBS group in Figs. 1–3). For chronic variable stress experiments (Fig. 4), a total of $n = 10$ rats ($n = 5$/group) was used. For functional analyses (Fig. 5), a total of $n = 28$ rats was used ($n = 6$–8/group). All rats were group-housed in standard cages in a temperature-controlled vivarium on a 12 h light/dark cycle with free access to food and water. After arrival at Ohio State, rats were acclimated to the lab environment for 2 weeks before entry into any experiments. All procedures were conducted in accordance with proposals approved by the Ohio State University Institutional Animal Care and Use Committee.

2.2. Stress protocols

2.2.1. Acute stress

Rats were placed individually into flat-bottomed plastic restrainers (diameter = 3.375; length = 8.5 in; Braintree Scientific, Braintree, MA) for 60 min immediately prior to intraspinal injection of glutamate. This acute stress paradigm causes a rapid increase in circulating corticosterone (CORT) and adrenocorticotrophic hormone (ACTH) (Girotti et al., 2006). Animals in the "no stress" control groups were relocated to a quiet room for an equivalent period of time.

2.2.2. Chronic stress

Using the above restraint device, rats were restrained daily for 2 h over a period of 2 weeks. In an attempt to avoid habituation caused by repetitive homotypic stress, rats were restrained at random times of day during the light–dark cycle (8 am–8 pm).

2.3. Excitotoxic injury

Rats were anesthetized with ketamine (80 mg/kg i.p.) and xylazine (10 mg/kg i.p.). Using aseptic technique, a dorsal midline incision was made between the shoulder blades, skin dissected, and trapezius muscle cut lateral to midline from C1/2 to T2. Underlying muscle layers were blunt-dissected to expose the spinous process from C3 to T1. A single level laminectomy was performed at C5 to expose the spinal cord. To target motor neurons in the ventral horn of the gray matter, stereotaxic microinjections were made 1.0 mm off the spinal midline and 1.5 mm deep in the dorsal–ventral axis. A beveled glass micropipette (30–40 μm diameter) tip was connected to a pneumatic picopump microinjection device (DKI, Tujunga, CA) to inject glutamate (500 μM/3 μl, Sigma–Aldrich, St. Louis, MO) or vehicle (PBS). Injections were made over 10 min then were left in place for 5 min following injection to allow solution to diffuse into the parenchyma and minimize leakage from the needle tract as the micropipette was slowly withdrawn over a period of 1 min. The injection site was marked with charcoal (Sigma–Aldrich) on the spinal cord surface. Muscles were sutured with Vicryl and the incision closed with wound clips.

2.4. Bromo-deoxyuridine (BrdU) injections

Rats received intraperitoneal injections of BrdU (100 mg/kg dissolved in sterile water; Roche Diagnostics, Indianapolis, IN) 2, 4 and 8 h post-injection of glutamate.

2.5. Behavioral testing

The paw preference test is sensitive to lesion-induced asymmetries in forelimb activity in rodents (Gensel et al., 2006; Jones and Schallert, 1992). A clear plastic cylinder (20 cm × 46 cm) placed on end provides a small but non-restrictive exploratory environment for rats. Spontaneous exploratory behavior was recorded for 3 min and slow motion high-resolution video playback was used to quantify paw placements against the cylinder walls. A preferential placement was recorded when either the left or right paw made contact with the cylinder wall for $\geqslant 0.5$ s without the other forelimb also making contact. Rats were tested before injury (or stress) to obtain a baseline score then again 3 and 7 days after intraspinal glutamate injection. All behavior was recorded by an observer blind to group designation.

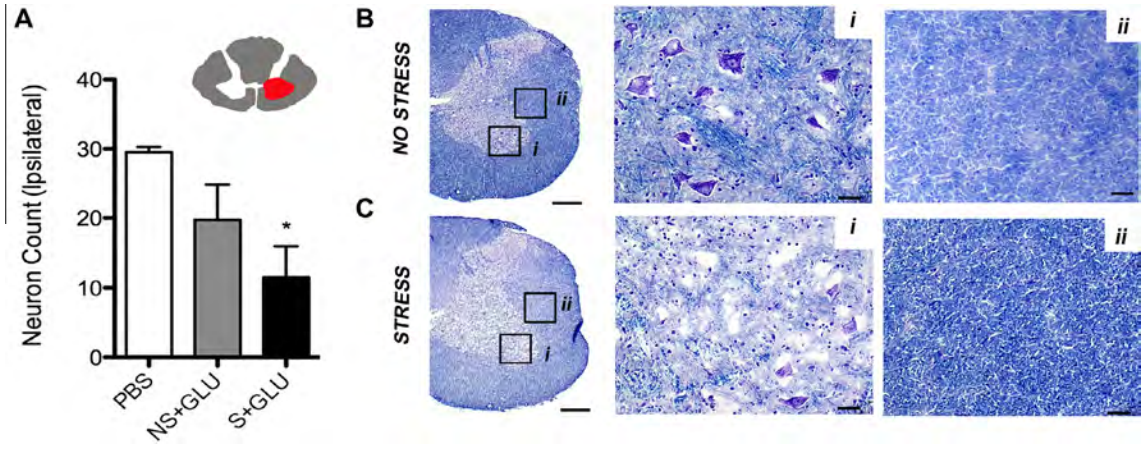

Fig. 1. Acute stress exacerbates motor neuron loss in a model of focal excitotoxicity. Glutamate (GLU; 500 nm/3 μl) microinjected into the ventral horn of the cervical gray matter killed 30% of neurons within 24 h, as compared to PBS controls (PBS). Acute stress (S; 60 min immobilization stress) prior to glutamate injections increased neuron death, killing more than twice as many neurons as glutamate alone (A; $^{*}p < 0.05$ vs. PBS). Neuron cell loss and atrophy is evident at the microinjection site (B, B_i) but surrounding tissue appears normal (B, B_{ii}) at 24 h. In acutely stressed animals, prominent neuron loss (C, C_i) is evident while adjacent white matter appears normal (C, C_{ii}). Data represent mean ± SEM, $n = 4$–8 rats/group.

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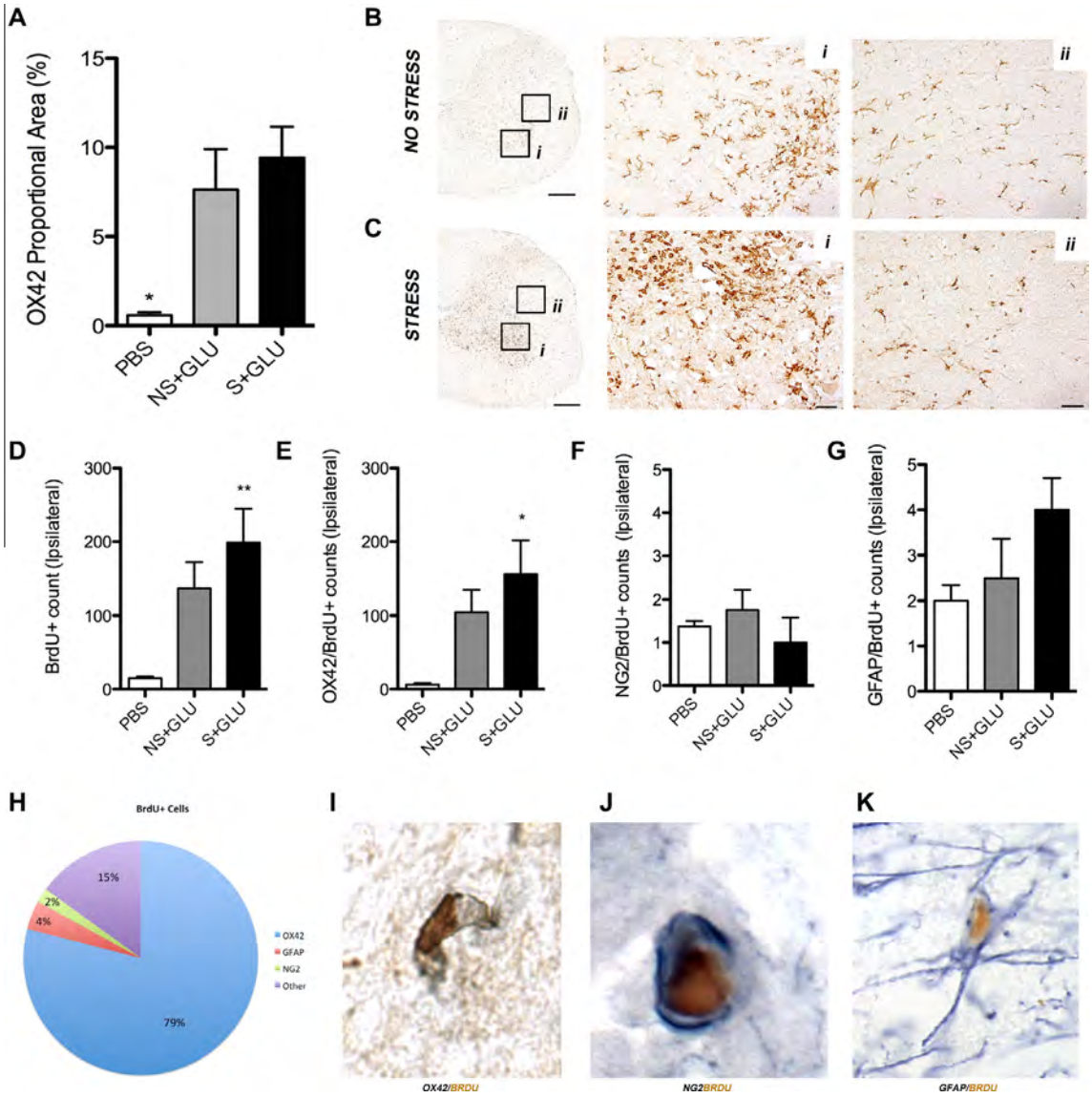

Fig. 2. Acute stress enhances microglial proliferation in a model of focal excitotoxicity. Glutamate injections activate microglia within the gray and adjacent white matter ipsilateral to the injection site at 24 h ($^{*}p < 0.05$ vs. NS + GLU and S + GLU; A–C). Morphological indices of microglial activation (A) were increased by glutamate but were only modestly increased by stress; however, a 10-fold increase in cell proliferation caused by glutamate alone (D) was significantly enhanced by stress ($^{**}p < 0.01$ vs. PBS and $p < 0.05$ vs. NS + GLU; D). Of the three glial subtypes that were examined, microglia proliferated the most in response to GLU alone (NS + GLU), or GLU and stress (S + GLU) ($^{*}p < 0.05$ vs. PBS, E). There was no effect of glutamate, with or without stress, on proliferation of NG2 + cell or GFAP+ astrocytes (F, G). In S + GLU group, microglia proliferation accounted for 79% of proliferating cells at the microinjection site, GFAP+ astrocytes = 4% and NG2+ cells = 2% (H). Other BrdU+ cells (15%) were not phenotyped. Representative images of proliferating OX42+(I), NG2+(J) and GFAP+(K) cells 24 h post-microinjections. Data represent mean ± SEM, $n = 4–8$ rats/group.

2.6. Tissue processing and immunohistochemistry

Twenty-four hours post-intraspinal injection, rats were given a lethal dose of ketamine (120 mg/kg i.p.) and xylazine (15 mg/kg, i.p.) then perfused transcardially with 100 ml of 0.1 M phosphate-buffered saline (pH 7.4) followed by 300 ml of 4% paraformaldehyde. After perfusion, cervical spinal cord tissue was removed then post-fixed for 2 h, followed by a rinse and overnight immersion in 0.2 phosphate buffer (PB) solution. Tissue was cryoprotected by immersion in 30% sucrose for 72 h. Spinal cord tissue was embedded in optimal cutting temperature compound (OCT; Tissue-Tek, VWR International, West Chester, PA) then frozen at −80 °C. Spinal cord cross sections (10 μm) were cut using a cryostat and thaw-mounted on SuperFrost Plus slides (Fisher Scientific, Houston, TX), then stored at −20 °C until use. To stain tissues for light microscopy immunohistochemistry, slides were first dried at room temperature then were rinsed with 0.1 M PBS followed by a rinse in 0.5 M Tris Buffer (TB) then a solution of 1.0% Triton-X/0.2 M PB. After overlaying sections with primary antibodies prepared in blocking solution, slides were incubated overnight at RT. Primary antibodies used to stain glial subsets were specific for rabbit-NG2 (1:400, US Biological, Swampscott, MA); mouse anti-CD11b for microglia and macrophages (1:2000, OX42 clone; Serotec, Raleigh, NC), mouse anti-glial fibrillary acidic protein for astrocytes (1:4000; Sigma–Aldrich) and mouse anti-BrdU (1:200 G3G4; Developmental Studies Hybridoma Bank (DSHB), Iowa City, IA). Myelin and cell nuclei were visualized by eriochrome cyanine and cresyl violet (EC/CV) histochemical staining.

For BrdU immunohistochemistry, 6% hydrogen peroxide was used to quench endogenous peroxidases followed by PBS rinses and incubation in HCl at 37 °C for 30 min. After rinsing in PBS,

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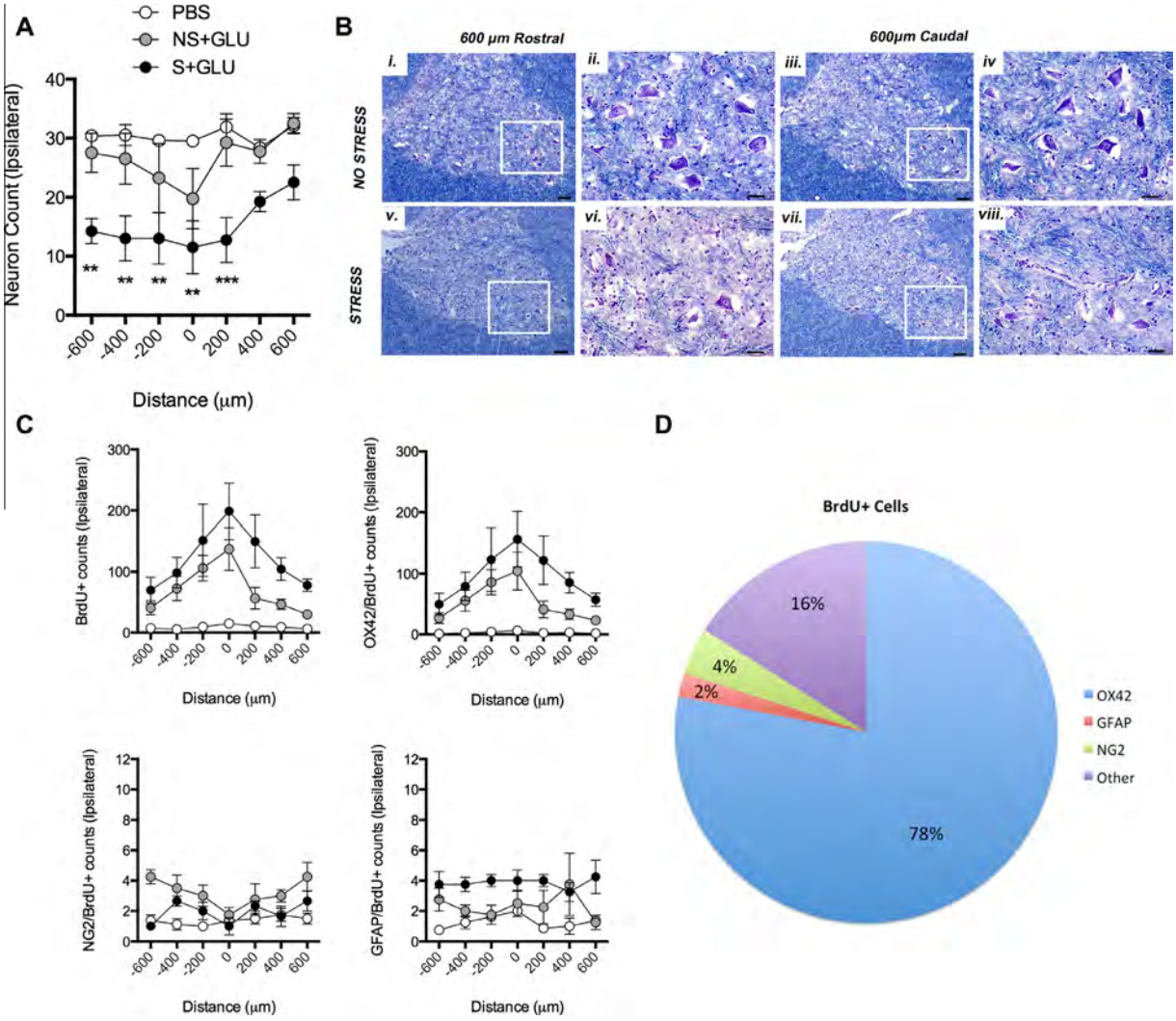

Fig. 3. Stress exacerbates the effects of glutamate on neurons and glia remote from the injection site. Neurons were counted at regular intervals rostral and caudal to the injection site over a total distance of ~1.2 mm. The neurotoxic effects of glutamate microinjection are restricted to within 200 μm of the injection site in non-stressed rats (NS + GLU; A, B). Conversely, acute stress (S + GLU) increased neuron killing over the full extent of analyzed tissue (A, B). Similarly, stress enhanced microglia proliferation at and beyond the microinjection site, while suppressing NG2 cell proliferation ($p < 0.05$) (C). GFAP+ astrocyte proliferation also was unaffected by stress (C). In S + GLU group, microglia proliferation accounted for 78% of all dividing cells (throughout the rostral-caudal length of the spinal cord), GFAP+ astrocytes = 2% and NG2+ cells = 4% (D). Other BrdU+ cells (16%) were not phenotyped. Data represent mean ± SEM, n = 4–8 rats/group; cells were counted at 200 μm regular intervals rostral and caudal to the injection site over a total distance of 1.2 mm. Group averages were analyzed with a 2-way ANOVA ($p < 0.05$) and a Bonferroni post hoc analysis was run to determine group differences at individual distances rostral and caudal to the injection site ($^{**}p < 0.01$ vs. PBS).

sections then were incubated in a blocking solution of 4% BSA prepared in 0.1% Triton-100 – PBS (PB^+) for 1 h. Tissue was subsequently incubated in mouse monoclonal anti-BrdU (1:200 in PB^+) overnight in a humid chamber at 4 °C. The following day, sections were rinsed and incubated with biotinylated horse anti-mouse IgG secondary antibody (1:400 in BP^+). Bound antibody was visualized by overlaying sections with Elite ABC (Vector Laboratories, Burlingame, CA) for 1 h then DAB substrate (Vector Laboratories) for 10 min. Sections were dehydrated then cover slipped.

2.7. Neuron and glial quantification

An investigator blinded to experimental conditions completed all quantitative analyses. Labeled cell profiles were manually counted in the ipsilateral side of transverse cross-sections by focusing through the tissue at 40×. To assess neuron loss, the number of cresyl violet (>30 μm diameter containing a nucleus and nucleolus) positive cells in the ipsilateral ventral horn (laminae VII–IX) was counted. Images were obtained from equally spaced sections at 200 μm intervals spanning a rostro-caudal distance of 1.2 mm (600 μm rostral and 600 μm caudal to the injection site). Overall, cell counts were obtained in 7 sections per animal. When quantifying cells double-labeled with BrdU and NG2, OX42 or GFAP, only cells with clearly visible staining around the circumference of the cell body (e.g., NG2+, OX42+) and a BrdU+ nucleus were included.

For data in Fig. 2A, the area of tissue occupied by OX42+ microglia/macrophages was quantified using techniques documented previously (Donnelly et al., 2009; Popovich et al., 1997). Briefly, using an MCID image analysis station, the ventral horn ipsilateral to the injection site was outlined and total area (region of interest/ROI) was quantified. Positively-labeled cells within that ROI were thresholded and the area occupied by stained pixels was divided by the total ROI to obtain a "proportional area" measurement.

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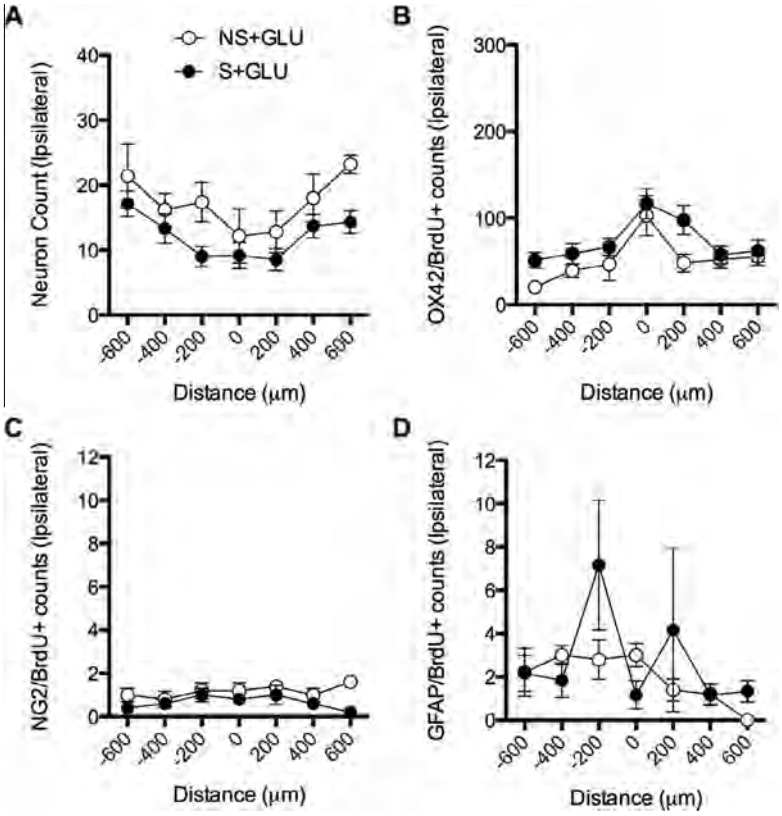

Fig. 4. Chronic stress increases spinal neuron excitotoxicity but not glial proliferation. Chronic variable stress (S + GLU) significantly increased neuron killing rostral and caudal to the microinjection site (A; $p < 0.05$ ANOVA main effect of stress). Conversely, chronic stress had no significant effect on proliferation of microglia (B), NG2+ glia (C), or astrocytes (D). Data represent mean ± SEM, $n = 5$ rats/group; cells were counted at 200 μm intervals rostral and caudal to the injection site over a total distance of 1.2 mm. Group averages were analyzed via 2-way ANOVA.

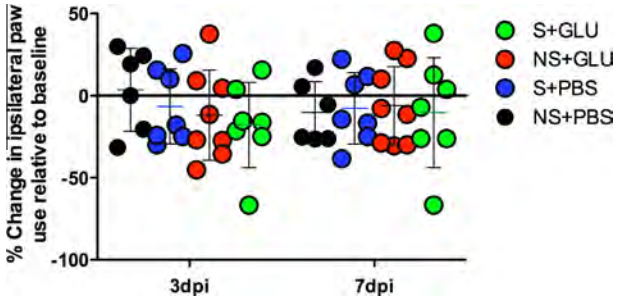

Fig. 5. Focal motorneuron excitotoxicity does not impair forelimb function. Rats receiving glutamate microinjections, with or without prior acute stress, were placed in a clear plastic cylinder and their paw usage was quantified pre-injury then again 3 or 7 days post-glutamate injection. Naïve rats show an equal preference for either limb as they explore the cylinder (solid line). A decrease in use for the limb ipsilateral to the injection site would be indicative of functional impairment. Although stress + glutamate injected (S + GLU, green) rats show a slight decrease in ipsilateral limb use at 3 dpi, this effect is not significant and is lost by day 7. Data represent mean ± SEM, $n = 6$–8/group; group averages were analyzed via 2-way ANOVA ($p > 0.40$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2.8. Statistical analysis

PRISM 5.0c (GraphPad, Inc., San Diego, CA) was used to complete statistical analyses. Cell counts obtained as a function of distance from the injection site were compared using 2-way ANOVA with treatment and distance as independent variables with repeated measures on distance from the injection site. Stress or non-stress control values were compared with PBS using two-tailed Student *t*-tests. An *a priori* alpha value of 0.05 was used for all analyses. Error bars in all groups depict SEM.

3. Results

3.1. Acute stress exacerbates spinal motor neuron excitotoxicity and microglial proliferation

The ability of stress to influence neuron survival and glial (microglia, NG2+ glia and astrocytes) activation was evaluated in a model of focal excitotoxicity. Glutamate (GLU) was microinjected into the ventral horn of cervical spinal cord gray matter then, 24 h later, surviving neurons were counted in lamina IX of the ipsilateral ventral horn (Fig. 1A, inset). Glutamate (no stress; NS) killed ~30% of neurons within 24 h (Fig. 1A and B). Acute stress (S; 60 min immobilization stress) prior to glutamate injection killed more than twice as many neurons as glutamate alone (Fig. 1A and C). Since PBS microinjections had no effect on neuron survival, with or without stress, all control data were collapsed into one group (PBS).

Microglia survey the intact nervous system and in response to cell death/injury, they proliferate and transform into phagocytes. To characterize the magnitude and distribution of microglia responding to glutamate-mediated neuron killing, with or without prior exposure to acute stress, tissues were labeled with antibodies specific for microglia (OX42) (Fig. 2). Enhanced microglial activation was evident ipsilateral to the injection site (Fig. 2A and B). Although the area of tissue occupied by reactive microglia did not increase in rats subjected to acute stress, it was obvious that

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stress affected both the density of labeling and the morphology of microglia (Fig. 2A–C).

To determine whether these qualitative differences in microglial reactivity were the result of enhanced cell proliferation, rats were pulsed with three injections of BrdU over a period of 8 h beginning 2 h post-glutamate injection. Tissues sections were prepared 24 h post-glutamate injection and were double-labeled with antibodies specific for BrdU and microglia/macrophages (OX42), astrocytes (GFAP) or NG2 glia (NG2). Single and double-labeled glia were counted within lamina IX as above. Intraspinal PBS injections had little effect on glial morphology or proliferation (not shown). Conversely, total cell proliferation increased ~10-fold after glutamate injection (GLU: 137 ± 35.04 BrdU+ cells vs. 14.75 ± 2.72 BrdU+ cells in PBS sections) (Fig. 2D). Stress significantly enhanced cell proliferation (S + GLU: 198.5 ± 46.13; $p < 0.05$ vs. GLU only) (Fig. 2D). Of the three glial subtypes analyzed, microglia proliferated the most in response to GLU alone or GLU + stress. Glutamate-mediated killing of neurons increased the number of microglia/BrdU+ cells ~17-fold as compared to PBS injection (104.3 ± 31.05 vs. 6.25 ± 1.88). Microglial proliferation was further increased by stress (Fig. 2E,H,I). In contrast, there was no obvious effect of glutamate, with or without stress, on proliferation of NG2+ glia or GFAP+ astrocytes (Fig. 2F–H,J,K).

3.2. Stress enhances neuronal toxicity and glial reactivity remote from the glutamate injection site

Acute immobilization stress was recently shown to enhance the efficacy and strength of glutamatergic synapses, in part by increasing multivesicular glutamate release (Kuzmiski et al., 2010). Accordingly, we predicted that the effects of acute stress on neuronal excitotoxicity would extend beyond the site of glutamate injection. To test this hypothesis, neurons were counted at regular intervals rostral and caudal to the injection site over a total distance of ~1.2 mm. As in Fig. 1, glutamate excitotoxicity was evident at the injection site with mild neurotoxicity evident within 200 μm proximal or distal to the injection. At distances >200 μm, neuron loss was rare, presumably because the concentration of injected glutamate decreases as it diffuses away from the injection site. In contrast, acute stress increased the rostral-caudal distribution of glutamate-mediated killing, with significantly more neurons dying over the full extent of analyzed tissue (Fig. 3A and B). Stress also enhanced microglial proliferation at and beyond the site of glutamate injection (Fig. 3C and D). Conversely, stress suppressed NG2 cell proliferation (Fig. 3C and D). GFAP+ astrocyte proliferation was unaffected by glutamate, with or without stress (Fig. 3C and D).

3.3. Chronic stress exacerbates spinal motor excitotoxicity but not glial proliferation

Brief periods of stress can enhance immune cell trafficking while prolonged or repeated bouts of stress can adversely affect immune function (Dhabhar et al., 2000; Dhabhar and McEwen, 1999). However, recent data indicate that chronic repeat bouts of stress can exacerbate neuroinflammation (Munhoz et al., 2006, 2010). As a comparator for the effects of acute stress, neuron survival and glial reactions were compared in spinal cord gray matter of rats subjected to a modified form of chronic homotypic stress. Recent data indicate that chronic stress can synergize with inflammation to kill nigral dopaminergic neurons (de Pablos, 2006; de Pablos et al., 2014). To determine if a different model of chronic stress has a similar potentiating effect on spinal cord motor neuron excitotoxicity and glial proliferation, glutamate was microinjected into the spinal cord of rats subjected to a chronic homotypic stressor (see Methods).

Again, neuron loss was significantly increased by chronic stress ($p < 0.05$; *2-way ANOVA*); however, the effects were reduced relative to those caused by acute stress (compare Figs. 1 and 4). Similarly, a slight increase in microglial proliferation accompanied the increase in neuron cell death but the effects of chronic stress were not significant for any glial subtype (Fig. 4B–D).

3.4. Focal glutamate excitotoxicity does not cause overt deficits in forelimb function

To determine whether the enhanced excitotoxicity caused by stress adversely affects forelimb function, we measured limb-use asymmetry using the "cylinder" task. Despite significant neuron loss caused by glutamate injection, gross measures of forelimb function were unaffected at day 3 or 7 post-glutamate injection, with or without stress (Fig. 5). Even though a modest (non-significant) increase in limb asymmetry was detected in stressed rats injected with glutamate (S + GLU), additional behavioral tasks (e.g., gross volitional locomotor function in the open-field, stereotypic grooming patterns in response to water placed on the snout) also failed to reveal deficits in forelimb function (data not shown).

4. Discussion

Stress activates the hypothalamic–pituitary–adrenal (HPA) axis leading to an increase in the synthesis and release of glucocorticoids (GCs) which are stress hormones that can modify neuron structure (e.g., dendritic arbors), neuronal metabolism and signaling, and electrophysiological indices of learning and memory (e.g., long-term potentiation and long-term depression) (Kim and Diamond, 2002; Kim et al., 1996; Pavlides et al., 1993). It is unknown to what extent stress and GCs affect the function and plasticity of neurons in the intact or diseased spinal cord, although there are reasons to suspect that spinal cord and brain neurons will respond differently to identical stressors.

Glucocorticoid receptors (GRs) are expressed in neurons and glia throughout the cervical, thoracic and lumbar spinal cord (Ahima and Harlan, 1990; Fuxe et al., 1985; Yan et al., 1999). However, the ratio of type I (mineralcorticoid) and type II (glucocorticoid) GRs varies in spinal cord as compared to the brain. Also, the relative sensitivity and ability of GRs to bind the nucleus to initiate gene transcription is reduced in spinal cord relative to brain (Moses et al., 1991, 1989; Patacchioli et al., 1998). Such differences may explain why neuroinflammation in the spinal cord is exacerbated, as compared to the brain, in response to identical experimental lesions (Batchelor et al., 2008; Schnell et al., 1999; Zhang and Gensel, 2014).

Here, using a novel model of mild excitotoxic lower motor neuron injury, we show that stress, regardless of duration, exacerbates intraspinal neuron death and inflammation. These effects of stress were not limited to the injection site; stress enhanced neuronal vulnerability throughout the rostro-caudal extent of the spinal cord segment into which glutamate was injected. Although we did not define the mechanisms underlying these cellular changes, in various models of cerebral ischemia and epilepsy stress and GCs are known to disrupt neuronal and glial glutamate metabolism leading to excitotoxic cell death and enhanced inflammation (de Pablos, 2006; de Pablos et al., 2014; Dinkel et al., 2003; MacPherson et al., 2005; Munhoz et al., 2010, 2006; Sapolsky and Pulsinelli, 1985; Sorrells et al., 2013, 2009). For example, GCs increase trafficking and synaptic surface content of AMPA receptors in hippocampal neurons (Groc et al., 2008). Stress and GCs also affect microglia and macrophages, which upon activation, can affect the sensitivity of neurons and glia to glutamate. In response

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to GCs microglia/macrophages synthesize more inflammatory cytokines including TNF-α and IL-1β (MacPherson et al., 2005; Munhoz et al., 2010; Sorrells et al., 2013). TNF-α increases neuronal sensitivity to glutamate by rapidly increasing surface AMPA receptors (Hermann et al., 2001). Similarly, IL-1β indirectly promotes glutamate-mediated killing of oligodendrocytes by inhibiting glutamate uptake by astrocytes (Takahashi et al., 2003). Via these diverse mechanisms, stress and GCs could increase vulnerability of neurons beyond the epicenter of the pathological stimulus (e.g., glutamate injection, trauma, infection, etc.).

In a pilot study, we tested the ability of RU486, a glucocorticoid receptor (GR) antagonist, to reverse the potentiating effects of stress on neuron killing (data not shown). Those data were inconclusive. In retrospect, ambiguous results may be expected since stress affects several neuron-intrinsic and extrinsic signaling cascades that are GR-independent. For example, stress enhances sympathetic nervous system activity and the subsequent release of catecholamines. Like GCs, signaling elicited by catecholamines, notably norepinephrine, can prime microglia, leading to enhanced synthesis and release of inflammatory cytokines including IL-1β (Johnson et al., 2005, 2013).

In the current model of intraspinal glutamate excitotoxicity, stress increased microglia proliferation but suppressed NG2 cell proliferation. The effects of stress on microglia are consistent with data from other reports (Nair and Bonneau, 2006); however, the suppression of NG2 cell proliferation was surprising, especially since we and others have shown that NG2 glia thrive in inflammatory lesions in the spinal cord (Busch et al., 2010; Kigerl et al., 2012; McTigue et al., 2001; Schonberg et al., 2007). A global suppressive effect of stress on NG2 glia is unlikely since repeated restraint stress was recently shown to enhance NG2 proliferation in the brain, specifically within the locus-coeruleus (Seifi et al., 2014). Given that this region of the brain is enriched with noradrenergic neurons, perhaps in the absence of pathology, catecholamines rather than GCs regulate NG2 glia in response to stress. Future experiments are needed to precisely define the mechanisms that differentially regulate glial responses to stress in the intact and disease or injured brain and spinal cord.

4.1. Potential implications for stress and glucocorticoids in spinal cord pathology

Since spinal cord pathology often culminates in neurological impairment and the development of neuropathic pain, affected individuals undoubtedly experience bouts of intense psychological stress that could exacerbate pathology, disease progression and the magnitude of neurological impairment. In animal models of amyotrophic lateral sclerosis, stress exacerbates neuron loss and neuroinflammation and reduces lifespan (Fidler et al., 2011). In models of spinal cord injury (SCI), systemic GCs rise quickly after injury and high levels are maintained indefinitely (Lucin et al., 2007; Zhang et al., 2013). SCI in humans elicits similar responses signifying that stress is a ubiquitous consequence of traumatic SCI (Cruse et al., 1993). Whether GCs or stress exacerbate spinal cord neuron loss and glial pathology after traumatic SCI is not known; however, preliminary data (unpublished) from our lab indicate that stress impairs spontaneous recovery of locomotor function. It is possible that GCs synergize with mechanisms underlying neuropathic pain (i.e., enhanced central sensitization) to impair recovery of function. In support of this hypothesis, data generated in a spinal learning paradigm indicate that activating nociceptors via electric shock increases glial-derived TNF-α in the injured spinal cord and impairs adaptive spinal learning and recovery of function (Grau, 2014; Huie et al., 2012). Electric shock, which is a significant stressor that can elicit activation of the HPA axis and release of GCs, also accelerates the onset and enhances the magnitude of neuropathic pain after SCI (Ferguson et al., 2012; Garraway et al., 2014). Undoubtedly, the degree to which stress or GCs affect cell survival, neuroinflammation or spinal plasticity will vary between individuals (e.g., genetics) and with respect to when an individual experiences stress and for how long the various cell types in the CNS are exposed to the effects of stress (Sorrells et al., 2013, 2009).

Our data indicate that excitotoxic neuron loss is exacerbated by either acute or chronic homotypic (restraint) stress. Importantly, neither neuron loss nor microglial activation was augmented further by chronic stress. In fact, the effects of chronic stress were reduced relative to the acute stress paradigm. The most likely explanation for this is that chronic stress caused habituation and desensitization of GR-dependent mechanisms (Herman, 2013). However, as described above, the effects of stress also may be GR-independent. It is also useful to consider our findings in the context of emerging data that indicate that stress and glucocorticoids (GCs) are not always immune suppressive or "anti-inflammatory", i.e., stress and GCs can enhance inflammation (Sorrells et al., 2009). For example, acute stress potentiates the cellular inflammatory response in a model of delayed type hypersensitivity (Dhabhar and McEwen, 1999). These immune potentiating effects of stress require adrenal hormones and also inflammatory cytokines (Dhabhar and McEwen, 1999; Dhabhar et al., 2000). Stress also stimulates inflammatory cytokine production in the brain (Munhoz et al., 2010, 2006). *In vitro*, TNF-α and IL-1β can stimulate microglia proliferation (Ganter et al., 1992; Merrill, 1991). *In vivo*, microglia proliferation is increased by acute (Nair and Bonneau, 2006) but not chronic stress (Tynan et al., 2010). The precise mechanisms underlying these distinct responses are not known. In our model, acute stress exacerbates neuron death and microglia proliferation. Above we outline a mechanism whereby microglia could exacerbate the effects of stress on neuronal excitotoxicity; however, we cannot rule out the possibility that microglia are "bystander" cells that are responding to the enhanced neuronal pathology caused by aberrant glutamate metabolism (see above).

In the current model of intraspinal excitotoxicity, stress consistently exacerbated excitotoxic neuropathology throughout a portion of the C5 cervical spinal cord; however, even one week after the onset of neuron loss, behavioral impairment was not evident. Muscles in the shoulder, upper forearm and lower forearm are needed to lift, aim and advance the paw. The neurons controlling these proximal and distal muscles are found throughout the cervical motor neuron column which extends from ~C2-T1 (McKenna et al., 2000; Tosolini and Morris, 2012). Thus, it is likely that even though stress exacerbates neuron killing throughout the C5 segment, too few motor neurons were killed to impair the muscles needed for gross forelimb movement. Plasticity in spared circuitry also could compensate for motorneuron loss in this model, just as it does in more severe models of traumatic SCI where only mild or transient deficits in motor function occur (Goshgarian, 2003; Webb and Muir, 2002). Still, there is little doubt that stress or GCs affect function in seemingly intact neurons or spared circuitry. In fact, even in naïve/intact rats, stress impairs normal motor function. A chronic cold water swim stress paradigm was able to adversely affect the ability of normal rats to perform multiple behavioral tasks including ladder walking, sticky-tape removal and gait analysis (Metz et al., 2001).

Endogenous GCs produced during periods of acute or chronic stress could exacerbate neuron pathology, enhance neuroinflammation and affect structural and functional neural plasticity. Synthetic GCs (e.g., prednisone), which are widely used in the clinic to reduce inflammation, could have similarly detrimental effects, especially in cases of brain or spinal cord trauma where synthetic GCs can easily bypass the normally restrictive blood–brain barrier (de Kloet et al., 1998). These data, together with decades of data

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produced in models of brain injury/disease, indicate that stress is a potent but underappreciated disease-modifying factor that can significantly affect outcome from diseases of the spinal cord (Maldonado Bouchard and Hook, 2014).

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