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Serotonergic agonists facilitate forelimb recovery in rats with cervical spinal cord injury

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Serotonergic agonists facilitate forelimb recovery in rats with cervical spinal cord injury

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in Physiological Science

by

Benita Mikyung Jin

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ABSTRACT OF THE THESIS

Serotonergic agonists facilitate forelimb recovery

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Benita Mikyung Jin

Master of Science in Physiological Science

University of California, Los Angeles, 2018

Professor Victor R. Edgerton, Chair

Serotonergic agents have been shown to improve the recovery of stepping ability in spinalized animals, but not yet of reaching and grasping ability. In the present study we tested whether buspirone, a serotonin 5-HT_{1A} receptor agonist, or fluoxetine, a selective serotonin reuptake inhibitor, would facilitate forelimb motor function recovery after a C4 bilateral dorsal funiculi crush in adult female rats. Following injury, there was a significant decrease in single-pellet reaching and ladder-rung walking performance in all injured rats. From 1-6 weeks post-injury, 31 rats were tested on these tasks with and without Buspirone 1-2 mg/kg; or Fluoxetine 1-5 mg/kg. Buspirone reaching and grasping success rates improved rapidly within 2 weeks post-injury and plateaued over the next 4 weeks of testing. Forelimb performance after buspirone treatment returned to sham levels within 2 weeks of buspirone withdrawal. Fluoxetine treatment resulted in a progressive improvement in performance over 8 weeks, but performance on the ladder test did not change. The improved accuracy of reaching and grasping and the increase in spinal motor evoked potentials demonstrate improved supraspinal-spinal connectivity and within and among spinal sensory-motor networks. Combined these data suggest that buspirone or fluoxetine treatment has therapeutic potential for functional recovery after a cervical spinal cord injury.

The thesis of Benita Mikyung Jin is approved.

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The masters thesis is a version of a manuscript in preparation for publication.

SIGNIFICANCE STATEMENT

These data demonstrate that monoaminergic neuromodulation can facilitate behavioral and electrophysiological adaptations that improves forelimb reaching and grasping of the rat following a bilateral, dorsal cervical lesion. Improvements occurred with buspirone (5HT agonist) or fluoxetine (5HT reuptake inhibitor).Given that the mechanims differ, their combined effects could be additive or synergistic, depending on the injury. Both drugs induced significant functional reorganization of supraspinal-spinal connectivity, demonstrating that modulation of serotonin function could be an effective,relatively non-invasive strategy to enhance forelimb recovery after SCI. As shown for lumbosaral networks controlling hind limb function, the present data show that the cervical networks can also be neuromodulated pharmacologically, as shown electrically (Slawinska et al., 2014) to improve forelimb function after a cervical injury.

INTRODUCTION

Cervical spinal cord injury (SCI) damages descending and ascending sensory-motor projections, significantly reducing motor ability and serotonin (5HT) in the spinal cord (Kiehn, 2006; Alam et al., 2017; Zhang, 2016). Subsequent 5HT receptor upregulation and 5HT level downregulation further contribute to the dysregulation between antagonistic muscles in tasks requiring coordination, eventually leading to limited movement or paralysis caudal to the injury site (Hayashi et al., 2017; Cabaj et al., 2017). Reversal of this 5HT loss pharmacologically can improve hindlimb and forelimb recovery in spinalized animals (Van den Brand et al., 2012; Scali et al., 2013). The present results demonstrate comparisons of a 5HT agonist and a reuptake inhibitor after cervical SCI on forelimb muscle coordination and recruitment, and its consequences on skilled tasks.

Serotonin is a monoamine neurotransmitter and a key role player in movement. Mainly originating from the raphe nuclei of the brainstem, 5HT descends in the white matter of the mammalian spinal cord before terminating in the dorsal and ventral horns (Takeuchi et al., 1982). In the rat spinal cord, 3-9 5HT cells/animal are distributed in the spinal cord with exception to cervical segments (Netwon et Hamill, 1988). These cells are localized to laminae VII and X in the gray matter (Netwon et Hamill, 1988).. Cortical application of serotonin, acting through 5HT1A receptors, expanded forelimb motor map expression (Scullion et al., 2013). Closer examination of cellular components of spinal cord circuitry show that serotonin modulates spinal pathways by controlling motoneuron and interneuron excitability and afferent transmission, suggesting interactions between supraspinal and intraspinal circuitry are critical to

smooth execution of movement and level of success (Schmidt et Jordan, 2000; Abbinanti et Harris-Warrick, 2012; Abbinanti et al, 2012).

The effect of 5HT agents on the spinal cord and movement can vary based on the targeted 5HT receptor and mechanism of action (Zhang, 2016). 5HT2A and 5HT7 agonists are sufficient to activate hindlimb spinal cord circuitry, while 5HT1A agonists like buspirone can potentiate the coordination during locomotion when added to spinal cord stimulation (Gad et al., 2017). 5HT1A receptor agonists like 8-OHT-DPAT significantly expand forelimb motor maps essential for skilled movements (Slawinska et al., 2014). Serotonin selective reuptake inhibitor (SSRI) can also act on specific 5HT receptors, particularly having a relatively high affinity for 5HT2B receptors. An increase in extracellular 5HT induced by fluoxetine is significantly attenuated when 5HT2B receptors are absent (Peng et al., 2014).

Here we use two 5HT agents – the SSRI fluoxetine and 5HT1A receptor agonist buspirone – that act on different 5HT receptors and regulator proteins to test the hypothesis that 5HT modulation can improve coordination of forelimb motor pools and facilitate reorganization of supraspinal-spinal connectomes that mediate forelimb skilled tasks. To evaluate this, in the current study, we investigated whether daily administration of buspirone or fluoxetine would facilitate forelimb funtional recovery, such as reaching and grasping, as well as locomotor function after a cervical spinal cord injury in rats. We found that either 5HT drug significantly increased reaching and grasping successes within 6 weeks post-injury. Buspirone improved extensor and flexor coordination during reaches, and dynamically altered spinal cord excitability for selected forelimb muscles. Overall our results suggest that daily buspirone or fluoxetine administration from the early stage of SCI can facilitate forelimb fine motor functional recovery in SCI rats, and thus suggesting a therapeutic potential for functional rehabilitation after cervical spinal cord injury.

RESULTS

Buspirone treatment after SCI improves forelimb reaching and grasping function

All rats were trained on a classical reaching and grasping task and randomly divided into three treatment groups (Buspirone, Fluoxetine, or Sham) after a dorsal funiculus crush injury at spinal level C4 (Stackhouse et al, 2008). This lesion effectively damages sensory ascending fibers and the motor descending corticospinal tract (dCST), diminishing control of skilled movements in the forelimb. Buspirone (1-2 mg/kg/day), Fluoxetine (1-5 mg/kg/day), or saline (1-2 mg/kg/day) was administered (i.p.) beginning 7 days post-injury. Animals were tested once every week on the reaching and grasping task and ladder-rung walking task. Buspirone improved the reaching and

grasping accuracy of the treatment group as early as 2 weeks post-injury (Fig. 1). Skilled reaching and grasping scores recovered to a slightly lower plateau bu remained significantly different from sham-treated animals up to 6 weeks post-injury. At 6-8 weeks post injury, Buspirone rats were switched to sham treatment to evaluate the chronic, lasting effects of the serotonin agonist, and the initially Sham rats were switched to a buspirone treatment to evaluate the acute effects of the serotonin agonist at a subacute phase of injury. After switching treatments, there was a significant decrease in reaching and grasping accuracy in Buspirone-to-Sham rats ($P < 0.05$). Successful reaching and grasping did not recover in Sham-to-Buspirone rats by 8 weeks post-injury.

Qualitative analysis of reaching and grasping components showed a similar pattern with a significant increase of normal movements with Buspirone and of abnormal movements in Sham rats (Fig. 2A,B,C,D). The reaching and grasping task was subdivided into seven segments (advance, digit extension, arpeggio, grasp, supination I, supination II and release) and quantified separately for both the Buspirone and Sham group. Figure 2 shows accumulative scores of sequential segments of the reaching and grasping task. The Buspirone rats showed marked improvement in digits extension, grasp and release functions from week 1 post-injury compared to Sham group rats. Sham animals sustained forelimb deficits, particularly during the grasping and retrieval stages of the reach, across time.

Electrophysologic Assessment of Spinal cord neural connectivity

We examined muscle recruitment during the task by recording EMG activity from selected forelimb muscles during the single-pellet task. There were significant differences between the EMG patterns that were elicited before and after injury, and those evoked after treatment (Fig. 3). Prior to injury, a distinct EMG pattern appeared among all selected forelimb muscles during the task. EMG bursts in biceps and deltoid muscles correlated to the gross lift of the forelimb, while EMG bursts in the pronator correlated to the rotation of the paw to grasp and retrieval of the pellet to the mouth. Non-injured animals performed this task with reciprocal activation of flexor and extensor muscles, with alternation between the extension and retrieval periods (Fig. 3A). Ablation of the dCST severely diminished the magnitude and duration of EMG bursts in all muscles with exception to the extensor at 1 week post-injury. SCI animals were unable to lift their dominant paw beyond their chin and maintained a slightly clenched fist during the task. The neuromodulatory effects of buspirone on reaching and grasping dynamically changed the EMG patterns from 1 to 2 weeks post-injury, demonstrating a clear change in the duration and timing of EMG bursts in forelimb muscles during the pellet task (Fig. 3B). Buspirone animals showed during EMG activity was elevated by buspirone in the deltoid, extensor, and flexor muscles during

the task. Differences in EMG patterns in sham- and buspirone-treated animals were further exaggerated at 6 weeks post-injury (Fig. 3C). In sham animals, EMG bursts in the extensor had shifted from the retrieval to the extension period. This shift in sham animals was consistent with a smaller lift and increased wrist extension following attempts to grasp and manipulate the pellet, resonating with work demonstrating that the extensor stabilizes the wrist during gripping (Mateo et al., 2015). Flexor activity did not recover in sham animals over time, in contrast to that of buspirone animals (Fig. 3B,C,D). Daily serotonin appliction following injury reorganized the strength of input to different motor pools, allowing dynamic recruitment and coordination among forelimb muscles to create a new strategy for the reaching and grasping task in buspirone-treated animals.

The EMG patterns during reaching and grasping at 8 weeks post-injury, despite the switch of treatments between buspirone- and sham-treated animals at 6-8 weeks post-injury, did not grossly change from 6 to 8 weeks post-injury (Fig 3D). In animals treated with buspirone at 1-6 weeks post-injury and switched to sham treatment at 6-8 weeks post-injury, the deltoid retained an elevated level of activity, and extensor and flexor muscles maintained antagonistic roles during the extension and retrieval stages of the task. On the other hand, sham-to-buspirone animals sustained greater activity in the extensor during the retrieval period compared to that of the extension period. Buspirone-to-Sham treated animals demonstrated similar EMG patterns at 6 and 8 weeks post-injury. The movement differed, however, in that the forelimb was more often extended to the side or above the pellet's location, resulting in less precision and fewer successful reaching and grasping attempts. Buspirone application to the initial Sham rats from 6-8 weeks post-injury did not dramatically alter muscle activity patterns during the single pellet task (Fig 3).

To quantitatively assess recovery of forelimb muscle recruitment and organization, we compared the EMG integral of muscle activity during the extension (Fig. 4D) and retrieval (Fig. 5F) periods of the single pellet task. As observed in the EMG activation patterns, cervical SCI decreased deltoid activity (Fig. 4A, 5A) and increased pronator activity (Fig. 4B, 5E) throughout the reach over time. In contrast, buspirone reversed these changes in the activity patterns of these muscles (Fig. 4A, B; 5A, E). Statistically significant decreases in biceps and flexor activity were observed in sham but not buspirone treated after injury further depicted *in vivo* functional network reorganization of the brain-to-motor pool projections after injury and treatment (Fig. 5B, C). Notably, switching treatments at 6-8 weeks post-injury elicited a greater variance of EMG integrals from 6 to 8 weeks post-injury in most muscles in all animals during the task (Fig. 4A, B, C, 5A, B,

C, E). Both injury and 5HT treatment exhibited neuromodulatory effects on both proximal and distal forelimb muscle activation during voluntary, skilled movement (Fig. 4, 5).

Pharmalogical modulation of spinal neural connectivity reflected in sMEPs

Spinal motor evoked potentials (sMEPs) were collected from rats at rest by applying low frequency stimulation (2Hz) to spinal levels C6 and C8 as demonstrated previously to characterize spinal circuitry and neuromotor function (Alam et al., 2015, 2017). sMEPs were dissected into early (1-10ms latency) and late responses (10-30ms latency). Early sMEP responses are likely reflective of direct activation of motoneurons and spinal reflex arcs (Fig. 6F) while late sMEP responses are derivative of secondary polysynaptic communication through spinal interneuron networks (Fig. 7F; Taccola et al., 2017). All animals generated sMEPs when stimulated with constant current protocol in the 100-1000 μA range while being awake and standing still. sMEP threshold per animal, or the current at which an animal first expressed sMEP responses in any of the selected forelimb muscles, did not significantly alter over time, injury, and treatment (data not shown).

We computed 3 parameters of sMEP responses using a custom-made MATLAB code with bandpass filtration, rectification, and additional TKEO filtration (Solnik et al., 2010): motor latency, area-under-the-curve (AUC), and peak-to-peak (P2P) amplitude. Motor latency in all recorded muscles across all timepoints did not significantly change despite injury and treatment, with variation of 1-2 ms difference from that of pre-injury. Forelimb circuitry in the spinal cord was still present after cervical SCI at spinal level C4 as evident from the presence of sMEPs after injury. Cervical SCI decreased the number of evoked early and late responses in response to stimulation across forelimb muscles excluding the extensor (Fig. 6B, 7B). Buspirone application increased the number of evoked responses, and by 6 weeks post-injury, had noticeably more evoked responses to stimulation (Fig. 6A, 7A). Nevertheless, injury increased the peak-to-peak amplitude (P2P; Fig. 6C) and EMG integral (AUC; Fig. 6D,E) of early evoked responses from the extensor and pronator muscles. Buspirone animals expressed lower levels of spinal excitability of extensor and pronator motor pools at 1-6 weeks post-injury (Fig. 6C,D,F). In contrast, buspirone animals expressed higher late responses from the biceps, flexor, and extensor motor pools than did sham animals (Fig. 7C,D,F). Cervical SCI and buspirone treatment significantly altered spinal excitability of motor pools caudal to the injury site and important for voluntary, skilled movement in the forelimb.

Buspirone treatment on forelimb locomotor function

To determine whether improvements on reaching and grasping translated into improvements on other motor tasks requiring fine motor control, injured rats were subjected weekly to the horizontal ladder rung walking task with unevenly spaced rungs. Foot faults were scored and summed on a 10-point system adapted from Metz et Whishaw (2009). We calculated the average number of foot faults over three consecutive sessions. In contrast to the reaching and grasping task, rats rarely produced errors in their forelimbs during locomotion (data not shown). Buspirone rats showed a significant decrease in partial foot placements at week 1 post-injury, but recovered by week 3 post-injury. Sham rats did not significantly differ from buspirone rats in locomotor performance at pre- and post-injury, showing that improvement in fine forelimb reaching did not transfer into improvements in locomotion (data not shown).

Influence of fluoxetine treatment after SCI on forelimb reaching and grasping function

In the first set of experiments, fluoxetine rats did not significantly improve post-injury despite increasing dosages (1-5 mg/kg/day). However, previous studies have shown that fluoxetine dose and administration can differentially promote or inhibit neuroplasticity (Pawluski et al., 2014; Bianchi et al., 2009). Accordingly, we performed a second set of experiments testing the administration of a higher fixed dosage of fluoxetine (5 mg/kg/day) on SCI rats (sham, n=10, fluoxetine, n=10). Fluoxetine rats with the fixed dosage significantly improved in the reaching and grasping task by week 1 post-injury (Fig. 8). Unlike that of the buspirone rats, fluoxetine rats with the higher fixed dosage did not reach a plateau ,but continued to improve over time. Sham rats slightly recovered to a higher plateau before decreasing in reaching ability at 8 weeks post-injury.

Qualitative analysis of reaching and grasping components in rats with higher, fixed dosages of fluoxetine showed a similar pattern to that of buspirone rats, with a significant increase of normal movements in Fluoxetine rats and of abnormal movements in Sham rats (Fig. 9). Fluoxetine improved fine movements throughout the reaching task, particularly during the retrieval stages of the reach, while sham animals sustained forelimb deficits across time.

DISCUSSION

The present results suggest that serotonergic modulation can improved forelimb reaching and grasping after ablation of the dCST . Using the partial $5HT_{1A}$ receptor agonist buspirone, we demonstrate that there is a muscle recruitment paradigm during reaching and grasping that is diminished and disorganized after injury, butreorganized within 2 weeks post injury after continuous serotonin application. Interestingly, serotonin withdrawal after 6 weeks of daily

application removed the benefits seen in forelimb movement after injury, suggesting that the benefits reflect a series of acute changes in the intraspinal circuitry over a chronic period of time. Acute changes in the spinal cord circuitry facilitated by buspirone lasted for at least 12 hours, but not for 2 weeks post-administration. Multiple behavioral and phsiological measures demonstrate functional reorganization of networks projecting to different motor pools, particularly those projecting to the more distal flexor and extensor muscles. Buspirone seems to have mediated an improvement in all kinematic phases of reaching and grasping. The present data suggest that the coordination of activity patterns among spinal motor pools of forelimb muscles are improved with buspirone and is a crucial component of skilled motor tasks. Although similar levels of recovery of forelimb function occurred with administration of the SSRI, fluoxetine, the improvement was incremental over time, reaching pre-injury success levels after after 8 weeks of treatment.

Motor evoked potentials can serve as a physiological biomarker of motor pool specific network reorganization

We propose that changes in the early and late sMEP responses in the presence of serotonergic modulation reflect the changes in the functional network connectivity that is mediated via a wide range mono- and polysynaptic circuits to different motor pools. Multiple physiological measures observed in EMG amplitudes and patterns among motor pools when perfoming the motor task and changes in well-controlled stimulation conditions in which amplitudes of MEP's to multiple motor pools are recorded in awake rats during motor tasksshow that there is substantial reorganization of neural networks that functionally connect supraspinal-to-spinal motor pools. These measures of functional reorganization occurred as acute and as chronic responses to serotonergic modulation. An obvious question is, what are the underlying anatomical events that enables these rather robust and dynamic functional reorganizations. It seems highly unlikely that the usual anatomical strategies used to verify these dynamic and functional changes will provide most of the changes observed here.

Potential mechanisms of monoaminergic modulation of cervical networks

We also showed that application of the SSRI fluoxetine, the most common treatment for major depression, also facilitates reaching and grasping ability in cervical SCI animals. Both 5HT agents, despite the difference in mechanism of action, within 6 weeks regained behavior similar to that of pre-injury in all stages of the task. The general improvement further implies that the 5HT agents potentiated reorganization among residual neuromotor networks that control

multiple motor pools rather than targeted a specific motor pool or population of interneurons after SCI.

Buspirone, more often known as Buspar, is primarily an anxiolytic drug with high affinity for 5HT1A receptors and weak affinity for 5HT2 receptors (Peroutka, 1988; Pecknold et al., 1994). Buspirone antagonizes dopamine D2 autoreceptors, further diversifying the several mechanisms in which buspirone could potentially modify monaminergic neurotransmission (Gobert et al., 1999). Low dosages of buspirone (1mg/kg) can decrease 5HT turnover without significantly decreasing motor activity, indicating the drug dosage preferentially stimulated somatodendritic 5HT1A receptors (Shireen et Haleem, 2005). Such stimulation has been suggested to produce inhibitory regulation on dopamine neurotransmission, potentially influencing motor behavior, and feeding circuits tied to motivation and emotion (Haleem et al., 2004).

Fluoxetine, widely marketed as Prozac, also has mixed influences on other systems and may potentiate circuits for certain behaviors by boosting neural plasticity. Accumulating evidence has demonstrated that fluoxetine promotes neurogenesis, gliogenesis, synaptogenesis, and cortical mapping in intact animals (Kodama et al., 2004; Hajszan et al., 2005; Kusakawa et al., 2010; Vetencourt et al., 2008). Researchers have translated these findings to facilitate functional recovery in stroke and degenerative disesases (Li et al., 2009; Cirrito et al., 2011). Theoretically the progressive improvement in function that we observed could be linked to an enhancement of 5HT reuptake among the surviving descending 5HT axons from the brainstem (Murray et al., 2010). Perhaps in a manner similar to the buspirone animals, fluoxetine may have increased serotonin communication and therefore coordination during single pellet task in fluoxetine animals. But even with these effects, given the progressive improvement in motor performance, there was a steady increase in supraspinal-spinal functional connectivity over the 8 weeks of treatment post-injury.

SUMMARY

Both serotonin agonists, despite upregulating the serotoninergic impact with different mechanisms, improved reaching and grasping performance.. Neither buspirone or fluoxetine displayed any signs of safety concerns – no tremors or dizziness were apparent.. The present experimental design does not provide any insight as to the roll of activity-dendent mechanisms in mediating the effects observed. Although the rats were not specifically trained to reach and grasp, they obviously performed similar movenents in their normal feeding behavior. Neither

was spinal epidural electrical neuromodulation used as an activity-dependent interventionas reported previously(). These activity-dependent variables are being addressed presently.. Recently, 5HT pharmacotherapy concomittant with exercise promoted dendritic plasticity and 5HT receptor upregulation in SCI rats has been reported (Ganzer et al., 2018). In a study of SCI paraplegic patients, it has been suggested that buspirone combined with transcutaneous electrical stimulation can restore voluntary control of a bilateral rhythmic stepping pattern (Gerasimeenko et al., 2015). All of these observations leave the possibility that improved fine motor function in the forelimb after SCI in adulthood in response to serotonergic facilitation could be attributed to be multiple mechanisms. This in itself provides a strong rationale for a more detailed understanding of the underlying explanations for the clear improvements in forelimb motor function in two qualitatively different responses to a relatively few exposures to two different monoaminergic pharmacological interventions.

MATERIALS AND METHODS

All experimental procedures were approved by the University of California Los Angeles Division of Laboratory Animal Medicine, and followed guidelines for working with animals (National Institutes of Health, Publication No. 86-23, revised 1985).

Animal subjects

Forty adult (2-3 months) female Long-Evans rats were evaluated for this study. All animals were housed in standard plexiglas cages (one animal per cage) in an animal colony lighted on 12 hour light/dark cycle. Training and testing were performed during the lighted portion of the cycle. Rats were placed on a food-restricted diet of 12-15 g of standard rat chow per day per animal to maintain 90% body weight. One week post-surgery animals were allowed free access to food. Animals were weighed three times a week to ensure adequate nutrition.

Surgical implantation of electrodes

Thirty-one of forty animals consistently demonstrated a success rate greater than 60% in the single-pellet reaching task, and fifteen of these animals were choosen for electrodes implantation as done in a previous study (Alam et al., 2017). Briefly, each rat was deeply anesthetized with a mixture of isofluorane and oxygen during surgery, and had its skull exposed for mounting a connector to access forelimb electromyograpy (EMG) and spinal epidural stimulation electrodoes connections. After hemostasis was achieved, two multi-socket plugs with pre-attached teflon-coated multi-stranded stainless steel wires (AS-631, 632, Cooner Wire, Chatsworth, CA) were

firmly anchored to the skull with six stainless-steel screws and dental acrylic. The EMG wires were threaded subcutaneously from the head-plug to five forelimb muscles (deltoid, biceps, pronator, flexor digitorum and extensor digitorum) in the prefered paw. During intramuscular wire implantation, the teflon coating was removed for 0.5-1.0 mm, and inserted into each target muscle using a pull-through technique (Roy et al., 1985). The wounds were closed with silk sutures. After surgery, the rats received subcutaneous injections of buprenorphine (0.05 mg/kg) for pain relief before recovering from anaesthasia and every 12 h for 3 days post-surgery, together with 0.02 mL antibiotic ampicillin and 0.6 mL of lactated Ringer's subcutaneously. The rats were kept in an incubator at ~37°C for 4 hours and then returned to their home cages. Recording sessions began 7 days postoperatively.

Spinal Cord Injury

The animals that underwent EMG and spinal epidural electrodes implantation also received a bilateral dorsal funiculi crush at cervical level C4, a month after electrodes implantation. Each rat was deeply anesthetized with a mixture of isoflurane and oxygen during surgery. A dorsal midline incision was made between the occipital bone and the dorsal edge of T2 vertebra, and underlying muscles were retracted. Following laminectomy of the C4 vertebrae, the dura membrane was cut to expose the spinal cord. An incomplete cervical SCI was induced by inserting the tips of a fine forcep 2 mm deep into the spinal cord parenchyma spanning the gap between the dorsal root entries (1.5 mm lateral to the midline) and down to the spinal canal, and keeping them closed for 20 seconds. Through this process the dorsal column fibers were effectively sectioned bilaterally (Alam et al., 2017). After surgery, the animals were given postoperative care and subcutaneous injections of the same content as those following electrodes implantation. Testing sessions commenced 7 days postoperatively.

Stimulation parameters

Monophasic and bipolar epidural stimulation at spinal levels C6 and C8 was used to evoke potentials in selected forelimb muscles of the preferred forelimb when animals were awake and at rest (2 Hz) at different current intensities using a constant current stimulator (Grass SIU5; Grass Instruments, Warwick, RI, USA) as previously shown (Alam et al, 2017). Preferred forelimb was determined by the single pellet reaching-and-grasping task. A period of rest was defined according to EMG activity in which forelimb activity was not above 2 standard deviations above baseline activity. Baseline activity was determined by taking the average of activity 100μs prior to a stimulation pulse. Spinal motor evoked potentials were divided into early (0-10 ms after pulse)

and late (10-30 ms after pulse) responses based on modification of characteristics of these potentials for the hindlimb muscles (Gerasimenko et al., 2006). The latencies, peak-to-peak amplitudes, and area-under-the-curve values were futher TKEO-filtered with a custom MATLAB code for the early and late sMEP responses. sMEP variables were normalized to pre-injury values per animal, and then transformed with a logarithmic scale.

Drug administration

Four treatment groups were used in this study, and were respectively assigned the treatments with (1) gradual buspirone administration (1-2 mg/kg/day,5 days/wk n=5), (2) gradual fluoxetine administration (1-5 mg/kg/day, n=5) (3) fixed fluoxetine administration (5 mg/kg/day, n=8) (Sigma-Aldrich), and (4) saline administration (1 mg/kg/day, n=13) as sham. Buspirone (Tocris) was dissolved in sterile water (1 mg/ml), sterile filtered and administered with intraperitoneal injection (1 mg/kg) into SCI rats according to treatment groups assignment. Fluoxetine was similarly prepared with sterile water, filtered, and administered with intraperiotneal injection (1-5 or 5 mg/kg/day). Normal saline (1 mg/ml) was similarly administered to that of buspirone. The rats received intraperitoneal injections of the same solution every 24 h. Recording sessions began 30 minutes after the injection.

Behavioral assesment

I. Reaching and grasping task

Prior to injury, all animals were trained on a single-pellet reaching-and-grasping task in a clear Plexiglas chamber (15cm x 30cm x 17.5 cm) in a manner similar to that reported by Whishaw et al. (1992, 1994, 1998). All animals were trained to grab and eat as many pellets (banana-flavored, sucrose 45 mg dustless precision pellets, Bio Serv, Frenchtown, NJ) from a platform for 10 min or until they achieved a maximum of 30 trials per day. The platform had two small indentations for the pellets. The indentations in the shelf were positioned so that the rats could not reach pellets with their tongue and therefore were forced to grasp them with their forelimbs (McKenna et Whishaw, 1999). For each rat, pellets were placed in the indentation optimal to the rat's natural preferred food position and limb as noted in the first week of training. Within three trials the animals developed a tendency to reach with one preferred forelimb directed at one indentation. Following habituation to the reaching chamber and food pellets, the rats were encouraged to move from the front to the back of the chamber after each trial via pellet drop in the back of the chamber. Moving back after each reach creates a natural separation between trials and encourages consistency in how the animal orientates and initiates itself to perform the next reach

(Whishaw et al, 1992). Ten of twenty rats demonstrated consistent reaching for single pellets at the end of training, with at least a 60% success rate. Each animal was videotaped from the front while retrieving food pellets at 100 frames/second with a video camera (SIMI Reality Motion Systems, Unterschleissheim, Germany). A frame grabber was used to capture individual fields for analysis. A successful trial was scored when the rat was able to reach, grasp, and eat the pellet. Cases in which the rat made an error in reaching, grasping, or eating were scored as failures. The success rate was calculated by counting the number of successful trials and expressed as percentage of total trials.

II. Horizontal ladder task

All animals were trained to perform the ladder rung walking task (Girgis et al., 2007) on a horizontal ladder a week prior to surgery. The ladder (1m long) consisted of side walls made of clear Plexiglas and fitted metal rungs (3mm diameter) irregularly spaced at distances ranging from 1 to 3 cm. The entire apparatus was elevated 30 cm above the ground with a neutral start cage and refuge (home cage) at the end. Animals were trained prior to injury with rungs spaced 1cm from each other. Two templates of irregular rung patterns were alternated every other week during post-injury testing. Analysis focused on categorizing and counting steps from both forelimbs as (1) miss: the paw was placed in between two rungs and did not make contact with any rungs before falling in-between rungs and disturbing body posture and balance, (2) slip: the paw was initially placed on a rung, then slipped upon weight-bearing and fell in-between rungs and disturbed body posture and balance, (3) partial placement: paw was placed on a rung with either wrist, digits, heels, or toes, (4) correct placement: mid-portion of the paw was placed on rung with full weight support and digits curved around the rung. Success and failure rates were calculated by counting the number of steps per category and expressed as percentages of total trials.

Data analysis

Following EMG and stimulation electrodes implantation, all the rats (n = 10) were tested and filmed during single pellet reaching task and the horizontal ladder walking task for baseline measurements of success rates. One week post injury, each group of rats (n = 5, refered earlier) were tested and filmed on the single pellet reaching task and the horizontal ladder walking task for 8 weeks. The last 2 weeks of testing the groups were switched to each other treatments for crossover examination. Videotape footage of forelimb reaches of each rat was examined frame by frame to match to the components of the reaching-and-grasping task. Footage of rats crossing

the horizontal ladder was similarly analyzed to match the start and end of each trial. Five individuals reviewed the videotaped reaching and stepping sequences and visually counted the number of consecutive successful reaches or steps at each time point. EMG plots for every trial of each muscle were generated by a custom script (MATLAB, Natick, MA) based on synchronization of video and EMG recordings. All EMG signals were first rectified and bandpass filtered at 30-1000 Hz and amplified (1000x) using a multichannel analog amplifier (Differential AC amplifier Model 1700, AM-Systems Inc., Sequim, WA, USA). Signals were collected 10kHz with a custom designed software written in LabVIEW (National Instruments Inc., Austin, TX, USA). Time bins for each extension and retrieval period during the single pellet task were normalized to a period of 1 second by interpolation of the digitized data with the Forecast function in excel and a custom formula based on the synchronization of video and EMG recordings.

Histological Assessment

At the end of functional evaluation, animals were anesthetized with an overdose of combination of ketamine (75 mg/kg) and xylazine (5 mg/kg), and perfused with 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS). Brain stem and cervical spinal cord segments were removed and post-fixed in the same solution at 4°C overnight, followed by 30% sucrose solution. Coronal sections of the spinal cord (30 µm thick) and of the brain (40 µm thick) were cut with a cryostat, mounted onto SuperFrost Plus slides (Fisher Scientific, Springfield, NJ), and kept at -20°C until use. Spinal cord tissue was counterstained with Cresyl Violet and coverslipped with a mounting medium with DAPI (Vectashield) to evaluate the extent and localization of injury. The images were magnified (5x) and the area and volume of the dorso-lateral funiculi injuries was quantified using a Zeiss Axiophot microscope and Apogee KX-85 camera (Apogee Instruments Inc., Roseville, CA, USA). Detailed images were acquired using Image Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA).

PRV Injection Procedures in Rats treated with a Fixed Fluoxetine dosage

After the final session to assess forelimb reaching and grasping ability, the flexor digitorium muscle in each arm of some fluoxetine rats (treated at 5 mg/kg/day) were injected with PRV-152 at four sites surrounding the motor end plates (2.5 µl/site, total virus of 2.5×10^8 pfu/ml). After 4 days, the rats were perfused and the tissues processed as described above prior to slide-mounting. For PRV-152+ staining, slides with spinal cord (30 µm thick), brainstem (40 µm thick), and brain sections (40 µm thick) containing the somatosensory motor cortex of the forelimb area were thawed and pre-incubated in 0.1M TRIS-BSA buffer containing 0.3% Triton-X and 5%

normal donkey serum (Vector Laboratories, Burlingame, CA) for 1 hour. The PRV-152 strain used expresses the reporter gene for enhanced green fluorescent protein (GFP). Sections were then incubated overnight at room temperature with a chick anti-GFP polyclonal primary (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:500) in the same buffer. The slides were then rinsed before applying donkey anti-chick conjugated to Alexa Fluor 388 (Jackson ImmunoResearch Laboratories, West Grove, PA, 1:250) in the same buffer. Following final rinses, slides were coverslipped with a mounting medium with DAPI (Vectashield) and imaged as described above. Regions were identified according to comparisons made to the Rat Brain Atlas 4e (Paxinos et Watson, 1998).

Statistical analysis

Single pellet reaching and ladder walking success rates were evaluated by ANOVA tests. Temporal and spatial parameters of EMG patterns over time and across groups were respectively evaluated by Wilcoxon tests. Lesion dimensions were evaluated by ANOVA tests. Data was examined with a computerized statistical program (JMP10, SAS Institute Inc., Cary, NC). Significance levels were set at $p < 0.05$ for all comparisons.

FIGURES

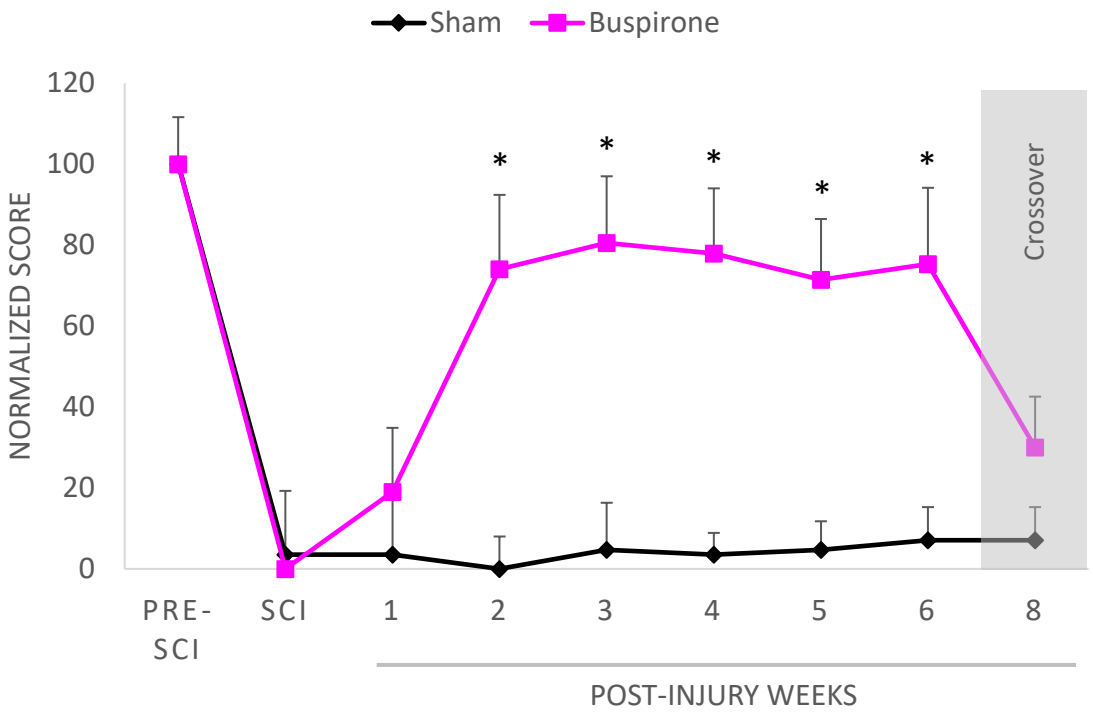
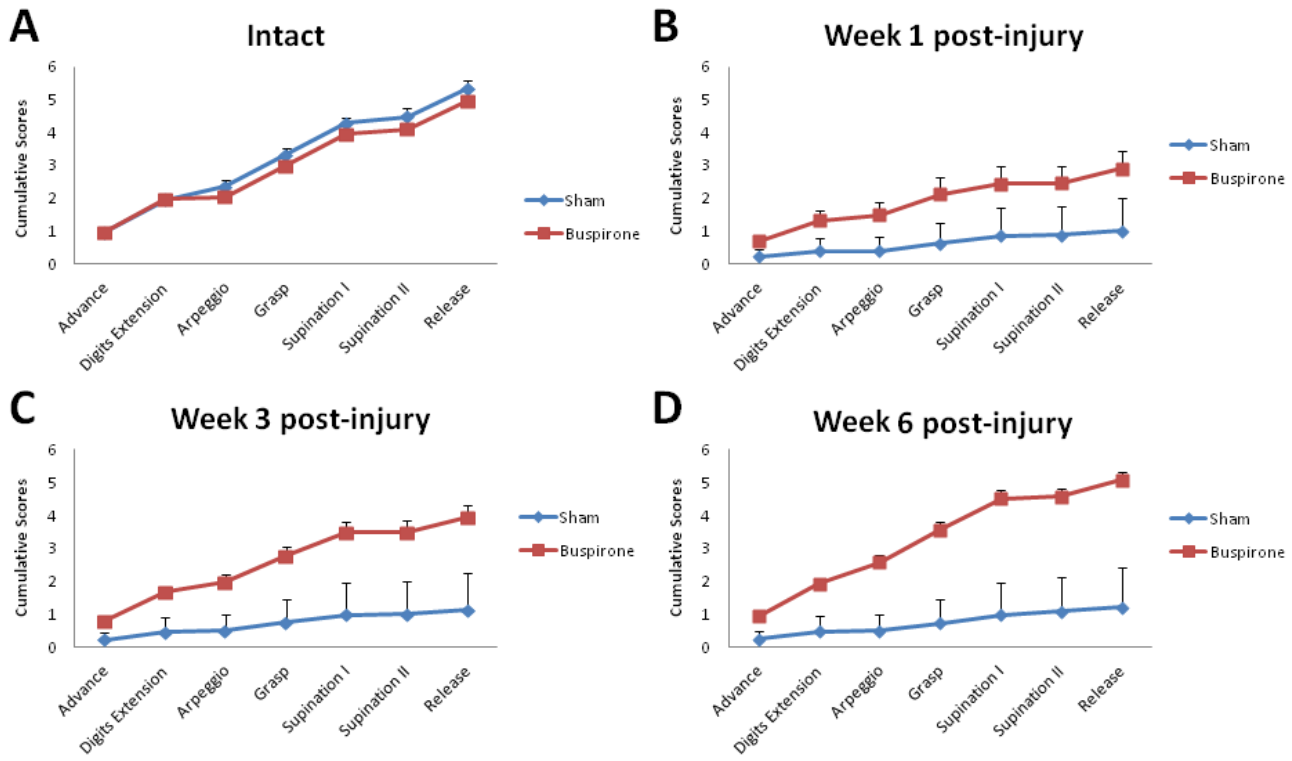


Figure 1. Normalized scores of reaching and grasping task with buspirone (n = 5), or saline (n = 5) intervention over time. Scores were calculated as percentages of successful reaches among 20 total reaches. From 6-8 weeks post injury, buspirone treatment was replaced with saline. All error bars are SEM. (*P< 0.05) between buspirone and saline (Sham) treatments for 2-6 weeks post-injury.



1, Normal movement; 0.5, abnormal movement; 0, absent movement

Figure 2. Qualitative scores of accuracies for the reaching and grasping task with and without buspirone **A,** before injury; and at **B,** 1 week, **C,** 3 weeks, and **D,** 6 weeks post-injury. Points were accumulated and averaged according to time point. All error bars indicate SEM.

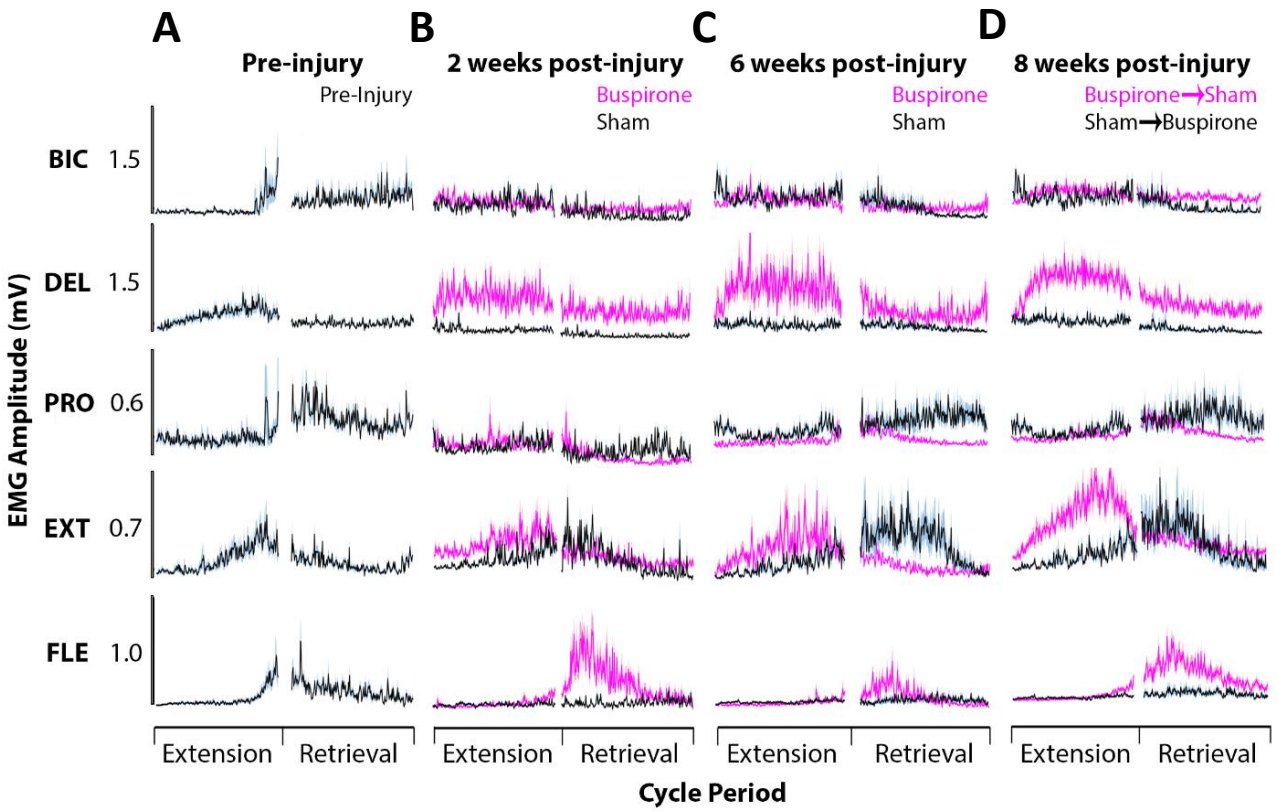


Figure 3. Normalized EMG activity of selected muscles in the dominant forelimb of four trained rats during the single-pellet task **A,** before injury (n=40 trials, black); and with (2 rats, magenta) and without (2 rats, black) buspirone at **B,** 2 weeks (n=20 trials), **C,** 6 weeks (n=20 trials), and **D**, 8 weeks post-injury (n=20 trials). Extension period was characterized by behavior as lift to arpeggio phases. Retrieval period likewise was defined as grasp to release phases. Extension and retrieval periods normalized according to behavior characterization per attempt. All shaded regions indicate SEM.

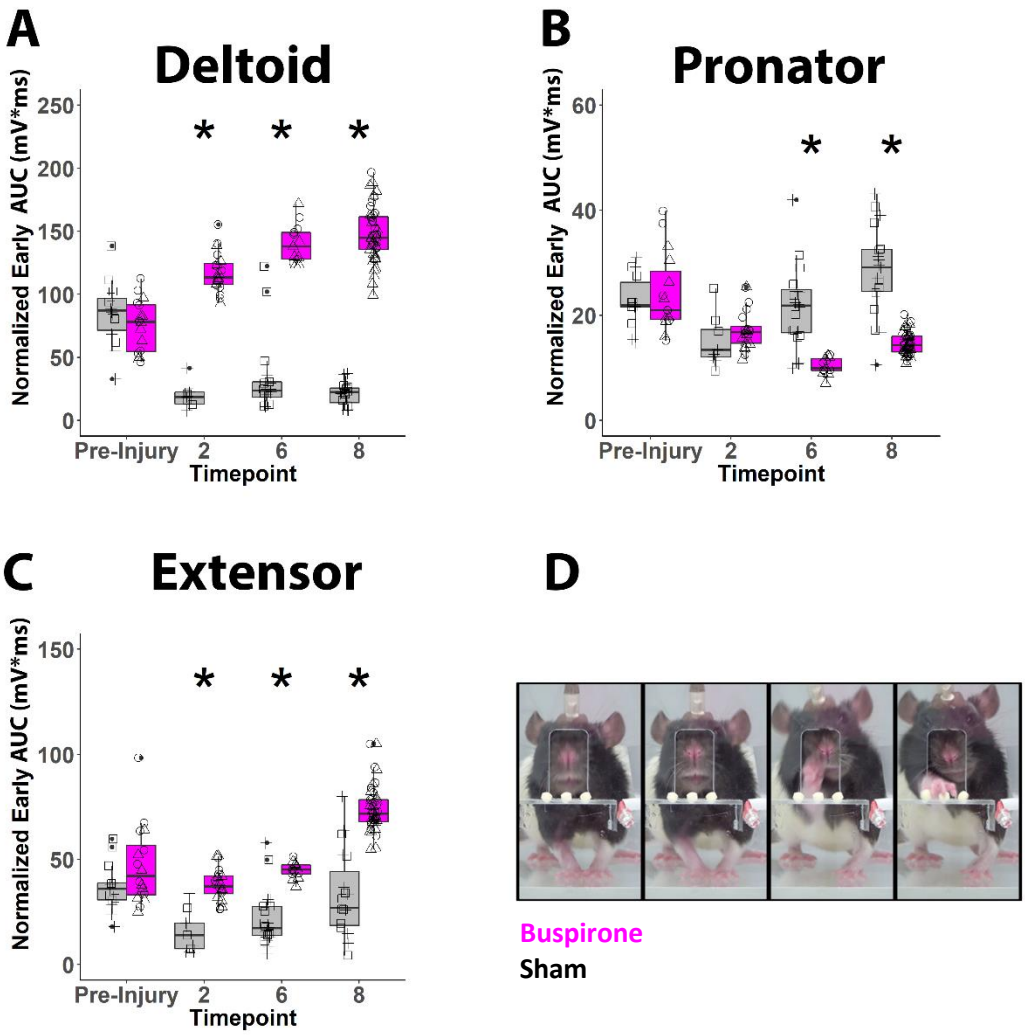


Figure 4. Normalized integrated EMG (iEMG) activity during the extension period in the dominant forelimb of four trained rats during the single pellet task before injury (n=20 trials, magenta; n=20 trials, gray); and with (2 rats, magenta) and without (2 rats, gray) buspirone at 2 (n=20 trials), 6 (n=20 trials), and 8 (n=20 trials) weeks post-injury. Significant changes found in the following muscles: **A,** Deltoid, **B,** Pronator, and **C,** Extensor. (*p = 0.0001, *Wilcoxon* test). Extension period was defined by **D,** reaching behavior (i.e. from lift to arpeggio segments of the task). Each marker shape corresponded to trials from one animal.

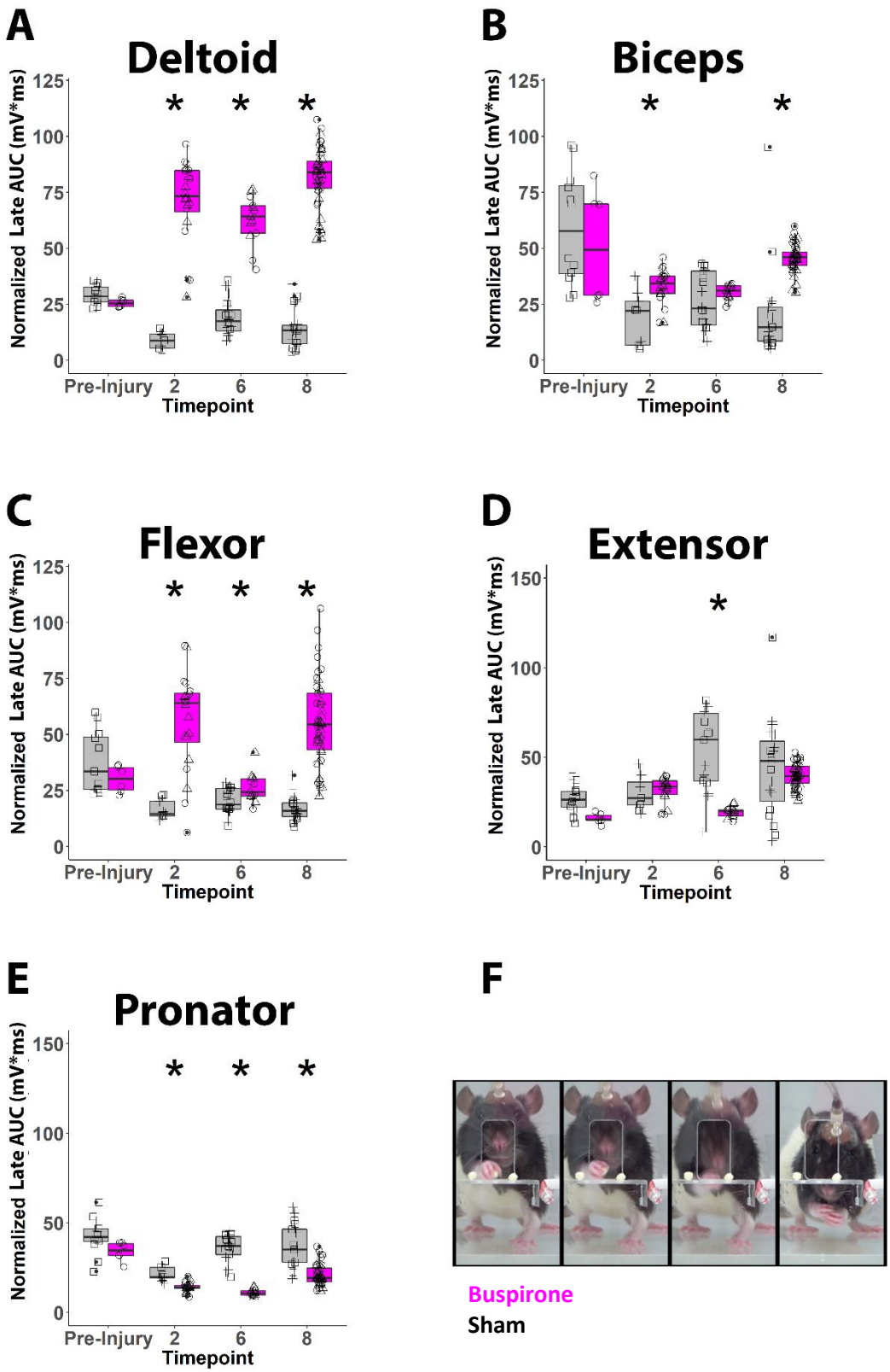
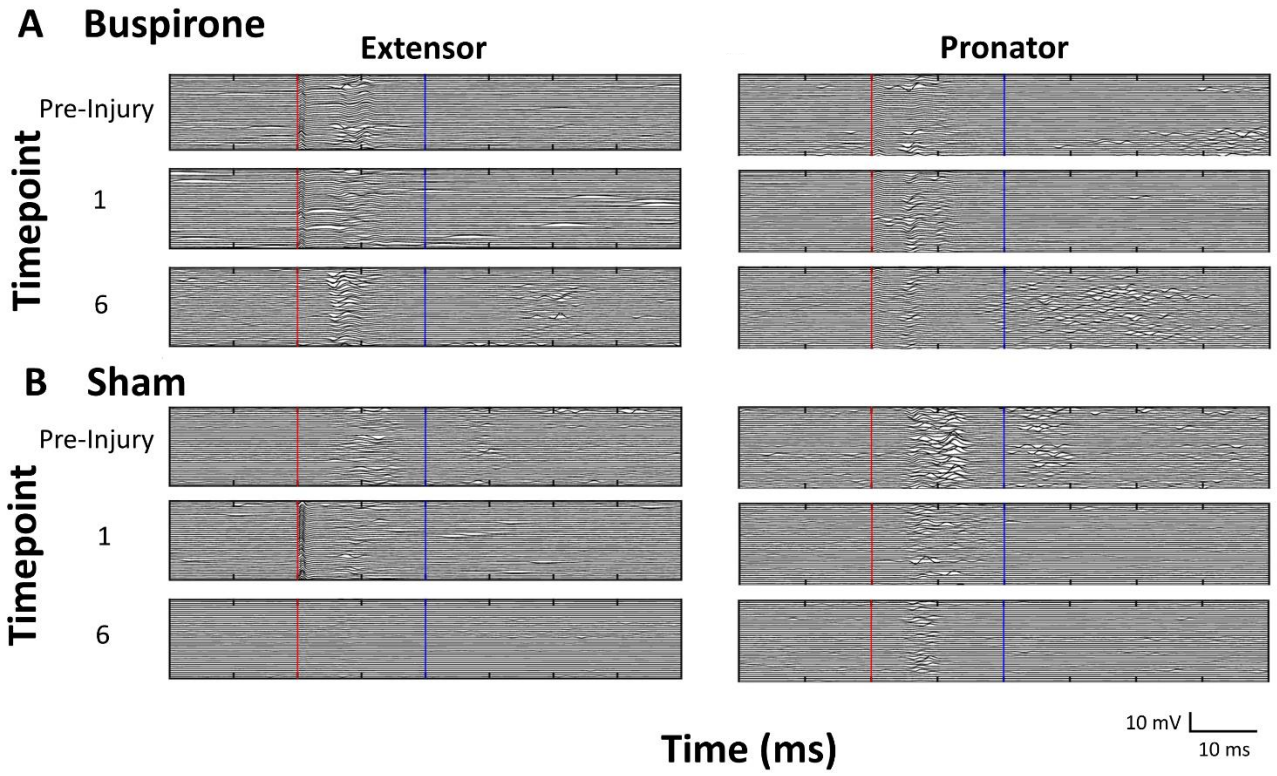
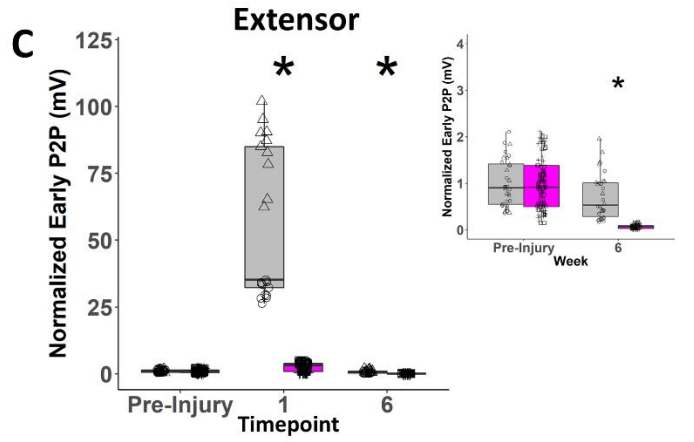
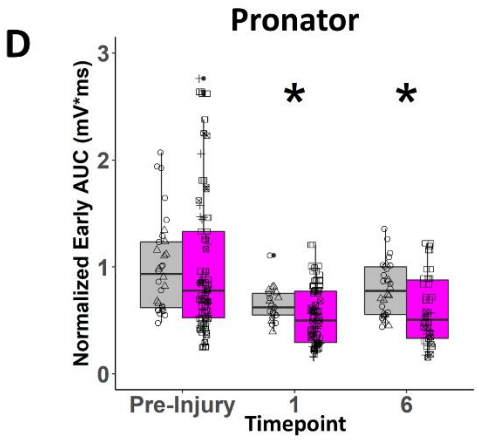


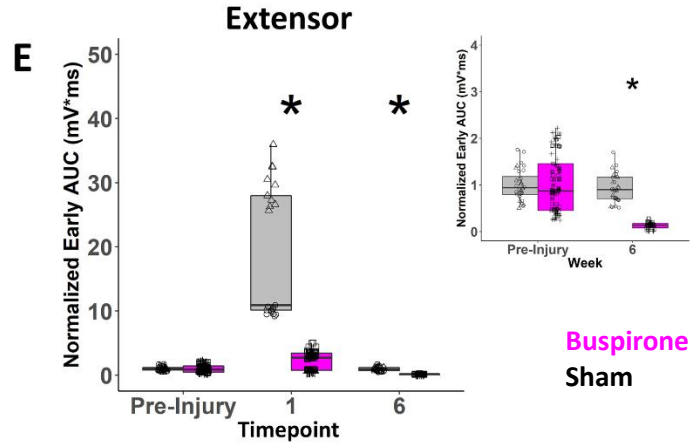
Figure 5. (*next page)* Normalized integrated EMG activity during the retrieval period in the dominant forelimb of four trained rats during the single pellet task injury (n=20 trials, magenta;

n=20 trials, gray); and with (2 rats, magenta) and without (2 rats, gray) buspirone at 2 (n=20 trials), 6 (n=20 trials), and 8 (n=20 trials) weeks post-injury. Significant changes found in the following muscles: **A,** Deltoid, **B,** Biceps (**p = 0.0043, *Wilcoxon* test), **C,** Flexor (**p = 0.0003, ***p = 0.0193, *Wilcoxon* test), **D,** Extensor, and **E,** Pronator (**p = 0.0001, *Wilcoxon* test). (*p < 0.0001, *Wilcoxon* test). Retrieval period was defined by **F,** reaching behavior (i.e. from grasp to release segments of the task). Each marker shape corresponded to trials from one animal.


A Buspirone
Extensor
Pronator
Pre-Injury
1
6
Timepoint
B Sham
Pre-Injury
1
6
Timepoint
10 mV
10 ms
Time (ms)


C
Extensor
Normalized Early P2P (mV)
Pre-Injury
1
6
Timepoint
Week


D
Pronator
Normalized Early AUC (mV*ms)
Pre-Injury
1
6
Timepoint


E
Extensor
Normalized Early AUC (mV*ms)
Pre-Injury
1
6
Timepoint
Week
Buspirone
Sham

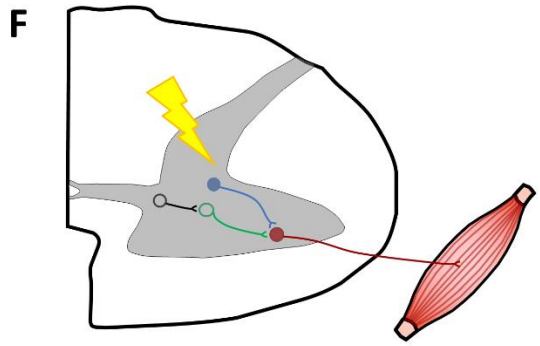
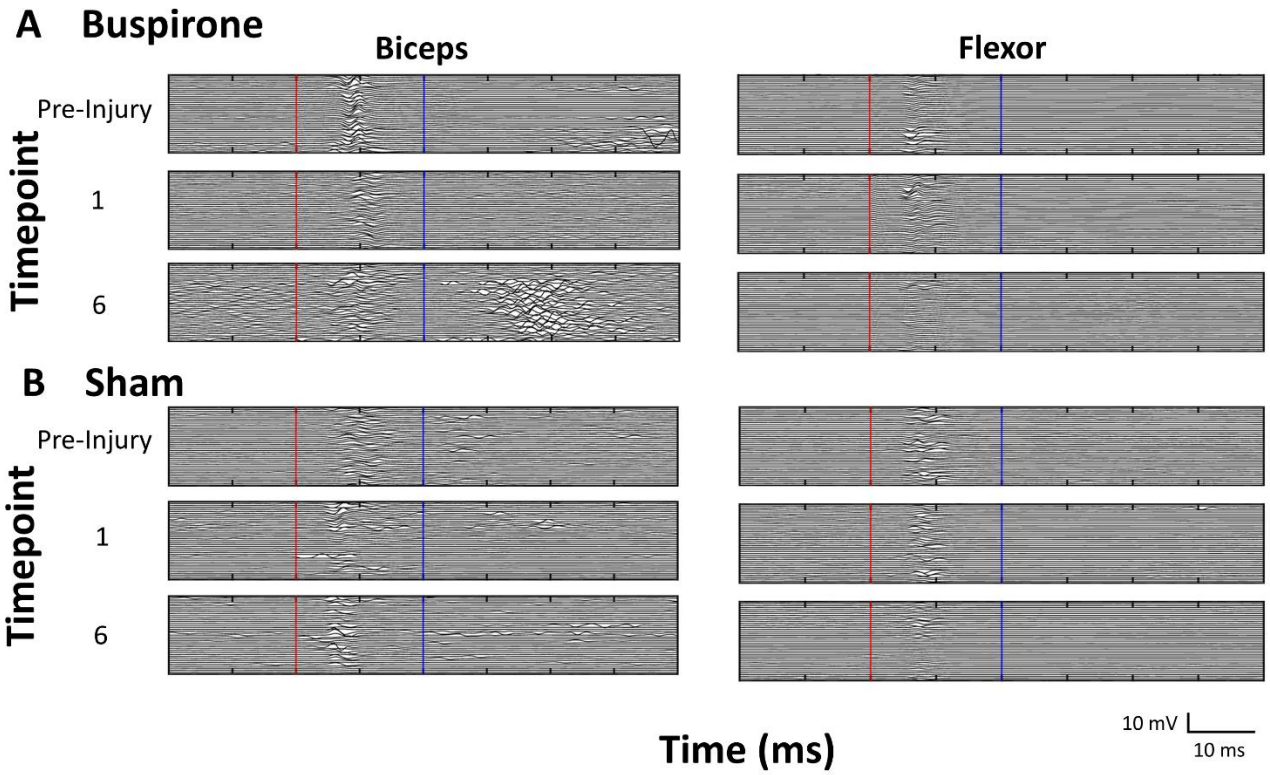
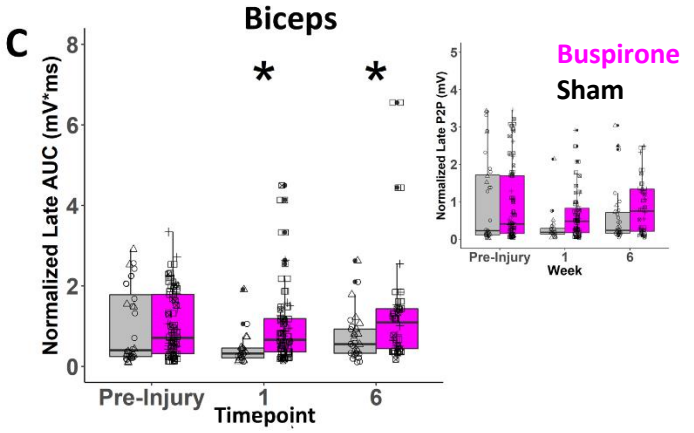
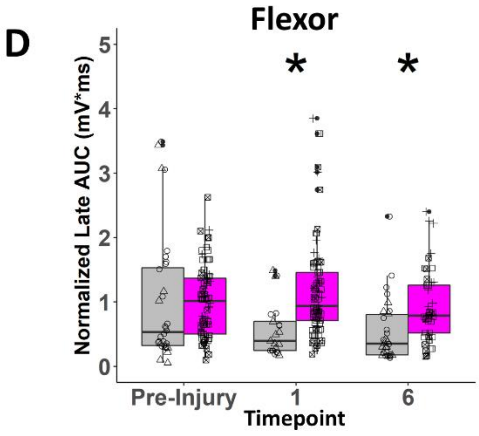
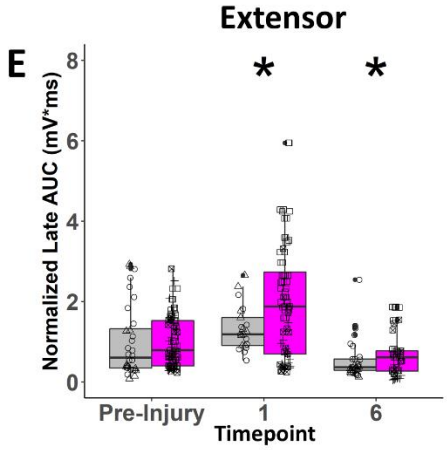

F

Figure 6. (*previous page)* Early spinal motor evoked responses (sMEPs) of selected forelimb muscles to epidural stimulation when the rats are at rest. Representative early (red to blue line) and late (blue line to end) responses (n=40) from the extensor and pronator muscles of sham- and buspirone-treated animals to monophasic stimulation pulses (red line) at C6-C8+ configuration for 2Hz, 500 µA at 6 weeks post-injury with **A,** buspirone, or **B,** sham treatment. Bouts of activity were removed by TKEO filtration. sMEPs were collected continuously for 30-40 seconds. Parameters were normalized per animal to their pre-injury responses, and then plotted on a log scale. Significant differences between the two treatment groups were found in early sMEPs for the parameters **C,** peak-to-peak amplitude, and **E,** iEMG in the extensor **D,** pronator muscle (***p* = 0.0450, ****p* = 0.0040, *Wilcoxon* test). (**p* < 0.0001, *Wilcoxon* test). **F,** General schematic eluding to the idea that early sMEPs reflect stimulation of direct spinal circuitry to motoneurons.


A Buspirone
Biceps
Flexor
Timepoint
Pre-Injury
1
6
B Sham
Timepoint
Pre-Injury
1
6
10 mV
10 ms
Time (ms)


C
Biceps
Normalized Late AUC (mV*ms)
Pre-Injury
1
6
Timepoint
Buspirone
Sham
Normalized Late P2P (mV)
Week


D
Flexor
Normalized Late AUC (mV*ms)
Pre-Injury
1
6
Timepoint


E
Extensor
Normalized Late AUC (mV*ms)
Pre-Injury
1
6
Timepoint

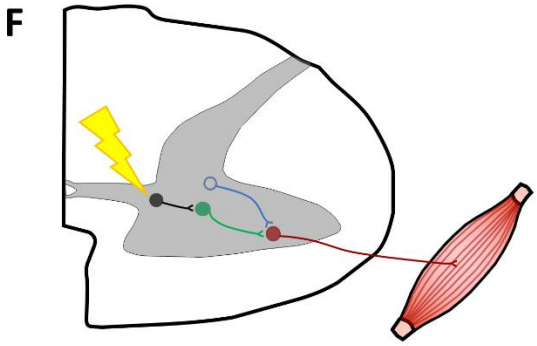

F

Figure 7. (*previous page)* Late spinal motor evoked responses (sMEPs) of selected forelimb muscles to epidural stimulation. Representative early (red to blue line) and late (blue line to end) responses (n=40) from the biceps and flexor muscles of sham- and buspirone-treated animals to monophasic stimulation pulses (red line) at C6-C8+ configuration for 2Hz, 500 µA at 6 weeks post-injury with **A,** buspirone, or **B,** sham treatment. Bouts of activity were removed by TKEO filtration. sMEPs were collected continuously for 30-40 seconds when the rats were at rest. Parameters were normalized per animal to their pre-injury responses, and then put on a log scale. Significant differences between the two treatment groups were found in late sMEPs for the parameters peak-to peak amplitude (***p* = 0.0054, ****p* = 0.0197, *Wilcoxon* test), and iEMG **C,** biceps (***p* = 0.0011, ****p* = 0.0106, *Wilcoxon* test), **D,** flexor (***p* = 0.0008, *Wilcoxon* test), and **E,** extensor muscles (***p* = 0.0301, *Wilcoxon* test). (**p* < 0.0001, *Wilcoxon* test). **F,** General schematic eluding to the idea that late sMEPs reflect stimulation of indirect spinal circuitry to motoneurons.

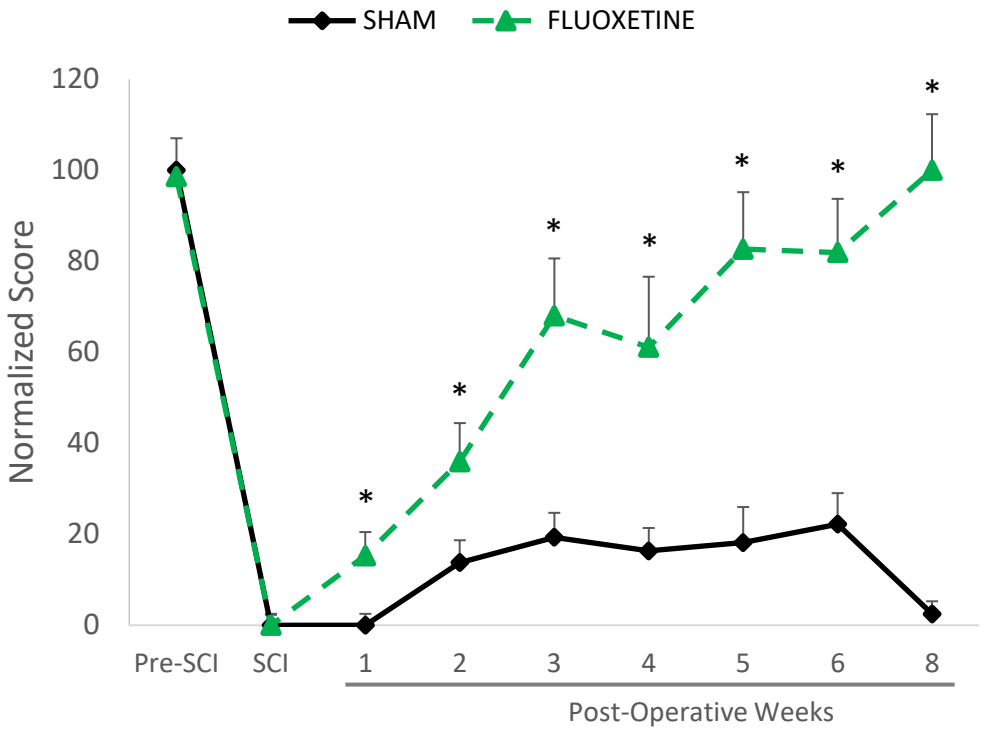


Figure 8. Normalized scores of Reaching and grasping task with and without fluoxetine (5 mg/kg/day) or saline (Sham) over time. Scores were calculated as percentages of successful reaches among total reaches. (*P< 0.05) between fluoxetine and saline treatments for 1-8 weeks post-injury. Error bars represent SEM.

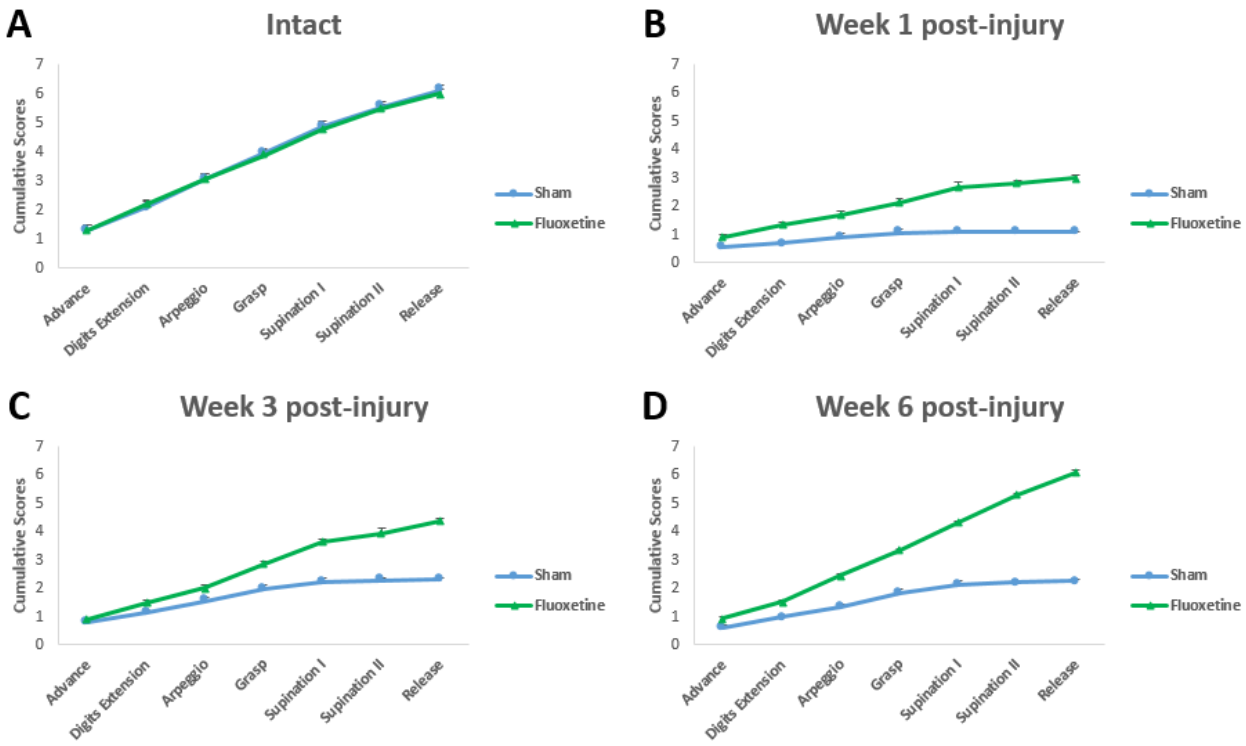


Figure 9 Qualitative score of accuracy for the reaching and grasping task with and without fluoxetine (5mg/kg/day) **(A)** before injury; and at **(B)** 1 week, **(C)** 3 weeks, and **(D)** 6 weeks post-injury. Points were accumulated and averaged according to time point. All error bars indicate SEM.

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