Serotonergic Modulation of Spinal Circuitry Restores Motor Function after Chronic Spinal Cord Injury

Sarita Walvekar,[1,2] Robert B. Robinson,[1] Hailey M. Chadwick,[1] Rebecca M. Burch,[1] Hanzhang Ding,[1] Steve I. Perlmutter[1] and Samira Moorjani[1,*]

[1]Department of Physiology and Biophysics, University of Washington, Seattle, WA 98195, USA

[2]Present Address: Cleveland Clinic Lerner College of Medicine, Case Western Reserve University, Cleveland, OH 44106, USA

*Corresponding Author: Samira Moorjani, 1705 Northeast Pacific Street, Health Sciences Building G-424, Box 357290, Seattle, WA 98195, USA; Phone: (206) 221-5338; E-mail: moorjani@u.washington.edu.

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Abstract

Upper limb motor recovery is a priority for chronic spinal cord injury (SCI) patients to improve functionality and quality of life. Treatment options available for acute SCI provide limited benefit to those with persistent motor symptoms years after injury, creating a need for interventions to target chronic motor damage.

Our therapeutic approach utilizes quipazine, a serotonergic agonist, as a chemical neuromodulator to induce plasticity of spared motor pathways in a rat model of chronic SCI. Therapy consisted of quipazine delivery via microinfusion pump and physical training with a forelimb reach-and-grasp task.

We found that quipazine administration produced a significant improvement in motor performance over rats receiving physical training alone. These therapeutic gains persisted beyond the duration of quipazine administration during a four-week follow up period, producing a long-term improvement in motor performance. Notably, our intervention produced the greatest gains in rats with more severe injuries over those with moderate injuries. When analyzing reach vs. grasp as individual components of total motor performance, quipazine administration produced greater gains in both measures, compared with physical training alone.

Our results provide evidence for quipazine administration as a therapeutic intervention to promote long-term motor benefits in chronic SCI. Our data offers support for the role of quipazine as a modulator of the dysregulated serotonergic system in SCI, promoting functional recovery. These findings have implications for the future use of neuromodulator therapy in improving motor performance to address the needs of chronic SCI patients.

Introduction

An estimated 27 million people worldwide live with spinal cord injury (SCI; 1), which often results from trauma suffered during motor-vehicle accidents, falls, or sporting and recreational injuries (2). Among developed regions, annual incidence of traumatic SCI varies from 39 cases per million of the population in North America to 15 – 16 cases per million in Western Europe and Australia (3). SCI can lead to motor deficits in the upper and lower extremities as well as sexual, bowel and bladder dysfunction, depending on the location and severity of the injury (2, 4). Motor deficits after SCI range from weakness in the limbs, abnormal muscle tone and posture, disruption in movement synergies, spasticity, and loss of balance and coordination to complete paralysis (5, 6). Secondary health complications often develop over time due to low levels or the complete lack of movement, further compromising the quality of life and patient autonomy (7). Most spontaneous recovery is limited to the first few months after the injury (2, 4). Additionally, a limited number of therapies have been developed for acute SCI (8), but they offer vastly diminished to no benefits to patients with chronic injuries, creating a need for interventions that target chronic motor damage.

Due to the limited intrinsic capacity of the adult central nervous system for remyelination and regrowth of damaged axons, existing approaches to motor recovery after chronic injuries seek to leverage spared circuitry for reinforcing functionally-relevant neuronal firing patterns through targeted physical rehabilitation (2). Use-dependent movement interventions, such as robot-assisted body-weight-supported treadmill training (9-11) and constraint-induced therapy (12-14), have been widely explored as treatment options in this effort, but they typically—and at best—lead to partial recovery of motor function. Greater success has been achieved through hybrid approaches that combine use-dependent rehabilitation with epidural electrical stimulation (15-26). However, the therapeutic gains almost always rely on the continued delivery of stimulation. In a small number of studies, residual effects were observed (15, 19), but such benefits were appreciably smaller compared to those obtained in the presence of stimulation. This lack of endurance in (the extent of) therapeutic benefits has created a need for development of alternate strategies that produce more lasting changes through plasticity induction in damaged circuitry. Towards this effort, we describe a neuromodulatory intervention that relies on intraspinal delivery of serotonergic agonists below the lesion to restore motor function after chronic SCI.

Serotonin (or 5-hydroxytryptamine; 5-HT) is an important neuromodulator in the spinal cord (27). Serotonergic regulation of spinal-neuron excitability is critical for normal sensorimotor integration and movement production (28). The activation of serotonergic receptors promotes a general increase in motoneuron excitability through amplification of synaptic inputs, depolarization, and increase in firing rates (29). The excitatory effects of 5-HT on motoneurons is dependent on the identity, location and level of receptor activation (29-31). Nonetheless, serotonergic modulation of spinal circuitry provides a potential therapeutic avenue for motor recovery by reinstating the (disrupted) excitation–inhibition balance after SCI (32, 33).

Previous studies have shown that serotonergic therapies can promote motor gains after SCI (34-41). Serotonin-receptor agonists can induce stepping and rhythmogenesis in spinalized rats after thoracic injuries through activation of spinal locomotor networks (34-40). Acute administration of 5-hydroxytryptophan, which is a 5-HT precursor, was also found to evoke activity in phrenic motoneurons following a C1 – C2 transection (41). However, the role of serotonergic agonists in the context of cervical SCI remains largely uninvestigated. To dissect this role, we developed a hybrid intervention that combines

intraspinal delivery of quipazine, a broad-spectrum serotonergic agonist (42), with targeted physical rehabilitation in an adult rodent contusion model of chronic cervical SCI that predominantly damages the corticospinal tract (critical for control of voluntary forelimb movements; 43). Injured rats receiving the hybrid intervention showed remarkable improvements in forelimb reach-and-grasp motor performance. Importantly, the therapeutic benefits outlasted neuromodulator delivery, signaling induction of plasticity in chronically-damaged adult spinal circuitry.

Results

Experimental design. Experiments were conducted in 20 adult female Long-Evans rats. Rats were trained on a standard forelimb reach-and-grasp behavior (44-46), using operant-conditioning protocols (47), for food-pellet rewards. Briefly, animals were placed inside clear plexiglass arenas and trained to reach out through a slit in the arena, grasp a food pellet placed on a block outside the arena with their dominant forepaw, and bring the pellet through the slit back into the arena and then to their mouth.

Rats were assigned a reach-and-grasp performance score of 1 (or 100%) for successful completion of all the steps in the forelimb-motor sequence. If the rat was unable to touch the pellet, due to inaccuracy in aim or the inability to extend its forelimb far enough, it was counted as a "miss" and was assigned a score of 0. Partial scores were assigned for completion of some—but not all—steps in the motor sequence. To assign partial scores, the motor task was divided into 'reach' and 'grasp,' with successful completion of the individual components leading to a score of 0.50 on each (and a combined score of 1). Scoring on the reach was binary with rats receiving either 0 or 0.50, whereas scoring of grasp occurred on a quaternary scale (with rats receiving 0, 0.17, 0.33 or 0.50). For example, if the rat dropped the food pellet inside the arena while bringing it to the mouth, it received a reach score of 0.50 and a grasp score of 0.33 (bringing the combined score to 0.83). If the pellet, however, was dropped outside the arena, the rat received a reach score of 0.50 and a (lower) grasp score of 0.17 (bringing the combined score to 0.67). Lastly, if the rat was only able to touch the pellet (but not grasp it), it received a reach score of 0.50 and a grasp score of 0 (bringing the combined score to 0.50). Additional details of the forelimb-motor task and the scoring system can be found in Materials and Methods.

Training on the forelimb-motor task occurred 10X/week, with the sessions divided across the five weekdays. Each session, in turn, consisted of 20 trials, which were preceded by a short warm-up. When rats had attained an average weekly reach-and-grasp performance of 80% or higher on the forelimb-motor task (which typically took 1 – 4 weeks), they received a unilateral C4 – C5 spinal contusion injury on the dominant forelimb side (48). This type of injury produces a marked and persistent inability to extend the elbow, wrist and digits, consequently, resulting in impaired performance on the previously-mastered skilled reach-and-grasp behavior (44). During week 4 after SCI, rats were implanted with a biocompatible plastic cannula caudal to the lesion in the C7 spinal segment. The cannula was coupled upstream to a pre-programmed battery-operated microinfusion pump (49), which was implanted subcutaneously. Seven weeks after SCI, a pre-therapy assessment on the same task was performed to quantify the functional deficits produced by the cervical cord injury. Therapy was initiated during week 8 after injury, which represents a common time point for a chronic SCI model in rats (50), and was administered during weeks 8 – 13. Therapy consisted of targeted physical rehabilitation combined with delivery of quipazine or vehicle. Physical rehabilitation consisted of ten sessions per week of training of the impaired forelimb on the pellet-retrieval task and occurred during periods of ongoing neuromodulator/vehicle delivery. To eliminate biases in scoring, all training was performed by researchers blinded to the therapy of the rats. Quipazine/vehicle was delivered intraspinally below the lesion for 4.5 hours/day for a total of six weeks. After completion of therapy, rats continued to be tested in the forelimb-motor task for an additional four weeks (in the presence of saline) to assess retention of motor gains in the absence of the neuromodulator. A terminal surgery was performed after completion of post-therapy follow-up to assess the integrity and location of the cannula. A detailed experimental timeline is shown in Figure 1A.

Note that rats in both intervention groups received (re-)training on the reach-and-

grasp pellet-retrieval task during weeks 7 – 17. Training on pellet retrieval served as both targeted physical rehabilitation and a measure of behavioral recovery promoted by our interventions.

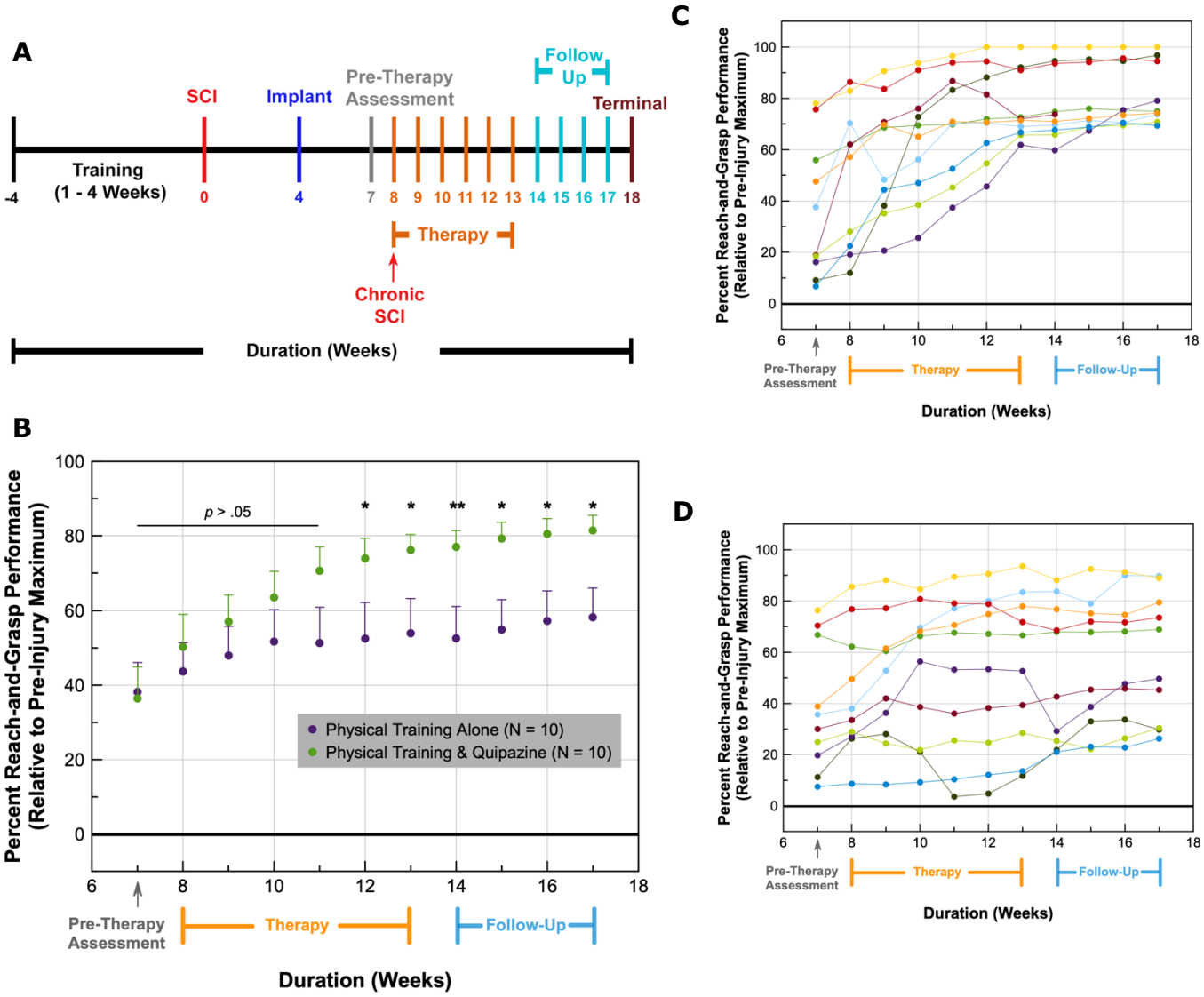

Figure 1. Intraspinal quipazine enhances motor recovery after chronic SCI. (A) Experimental timeline. (B) The reach-and-grasp score of injured rats, as a percentage of their individual pre-SCI maximum, was used to quantify the forelimb-motor performance before, (at several time points) during and after therapy. Data points represent weekly averages of all the rats in their respective intervention groups. 'N' indicates the total number of rats in each group. Error bars represent standard error of the mean. Only positive error bars are shown in the plot to reduce clutter. *p* values were obtained using paired-samples *t* tests. $*p \leq .05$; $**p \leq .01$. (C, D) Individual reach-and-grasp performance profiles of rats, whose means are shown in (B), that received quipazine (C) or vehicle (D) during therapy. Motor-recovery profiles of the rats are color-coded in the plots to reflect impairment-matching between the two groups.

Intraspinal quipazine improves motor-recovery outcomes after chronic SCI. Chronically injured rats received physical training combined with intraspinal delivery of (20 µg/week) quipazine or vehicle. Depending on their pre-therapy (or week 7) scores, animals were assigned to the two impairment-matched intervention groups. Rats were assigned to

the two groups based on their week 7 reach-and-grasp scores (*vs.* a randomization process) to account for variabilities in motor impairment across animals even when similar parameters were used for the contusion injuries. Larger-scale future investigations will be more amenable to randomization. Figure 1B shows the average weekly reach-and-grasp performance of rats in the two groups during the pre-therapy, therapy and follow-up periods. The reach-and-grasp performance of the injured rats is reported as a percentage of their individual pre-SCI maximum scores.

Injured rats that received physical training combined with intraspinal delivery of quipazine showed greater improvement in their forelimb-motor performance compared to rats that received physical training alone (Figure 1B). During the pre-therapy assessment, the reach-and-grasp performance of rats in the two groups was indistinguishable from each other ($t(9) = -0.845$, $p > .05$, paired-samples t test). Starting with the first week of the intervention, rats that received physical training and quipazine showed greater improvement in their performance scores relative to those that received physical training alone, and this difference in motor performance continued to widen across the therapy period. The motor-performance scores of rats in the two groups, however, were not significantly different from each other during the first four weeks of the intervention (*i.e.,* weeks 8 – 11). Beginning with the fifth week of the intervention (or week 12 after SCI), rats that received quipazine performed significantly better than those that received physical training alone. Importantly, therapeutic gains were maintained during the follow-up period (*i.e.,* during weeks 14 – 17) in the absence of quipazine. During the four-week follow-up period, the average forelimb-skilled performance of rats that received the combined intervention was 79% ± 4%, while rats that received physical training alone recovered 56% ± 8% of their pre-injury ability. The above changes, as well as those in the following sections, are reported as mean ± standard error (unless otherwise indicated).

Figures 1C and 1D show the individual motor-recovery profiles of rats that received intraspinal quipazine and vehicle, respectively. Motor profiles of rats that started at similar functional injury levels are represented by the same color in the two plots to illustrate differences in intervention-mediated recovery between them (*e.g.,* yellow curves depict the profile of the least injured rat in the two groups, while the darker-blue curves represent the most severely injured rat). Because rats in the two groups were impairment-matched, and they spanned a range of impairments, there was a high—but comparable—variability in motor performance within the two groups during the pre-therapy week (36% ± 9% and 38% ± 8% for rats receiving quipazine and vehicle, respectively). Variability in motor performance gradually decreased as therapy progressed in rats that received the combined intervention, with variability at its lowest during the follow-up period (79% ± 4%; Figure 1C). In contrast, variability in the physical-training-alone group stayed almost consistent during therapy and post-therapy periods, with an average of 56% ± 8% during (post-therapy) follow-up (Figure 1D). Notably, in the combined-intervention group, rats with higher injury severities (or lower reach-and-grasp scores) made more recovery during therapy that resulted in follow-up scores that were closer to the moderately-injured rats in the group, which ultimately reduced the overall variability over time (Figure 1C). This decreasing trend in performance variability was not seen in rats that received physical training alone (Figure 1D).

Intraspinal quipazine substantially augments forelimb-motor gains after severe cervical SCI. Figure 2A shows the average therapeutic gains (= mean post-therapy performance – pre-therapy performance) produced by our interventions. The mean post-therapy performance was calculated by averaging the reach-and-grasp scores obtained during weeks 14 – 17. Therapeutic gains were 2.4-fold larger with quipazine (43% ± 7%)

compared to physical training alone (18% ± 5%). We used a paired-samples *t* test to compare performance gains between impairment-matched rats, which revealed a significant difference between the two groups ($t(9) = 2.925$, $p = .02$; effect size = 1.57, very large effect). Details of effect-size calculations and thresholds used for interpretation of the magnitude of observed effects can be found in Materials and Methods.

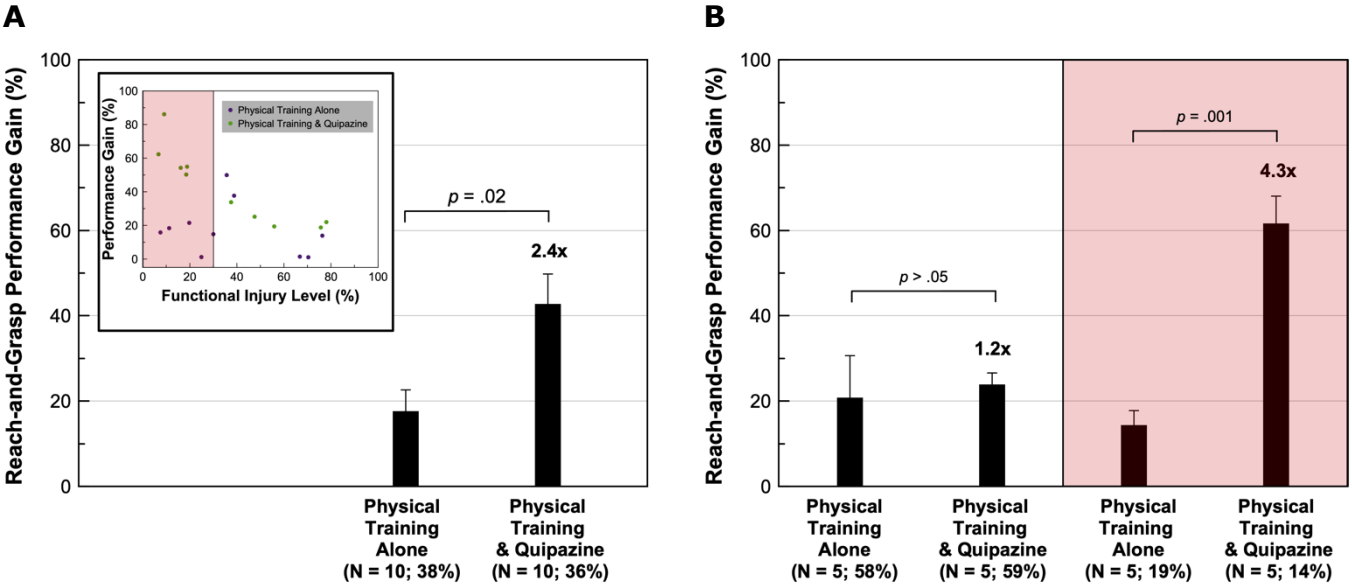

Figure 2. Intraspinal quipazine augments motor gains after severe SCI. (A) Bar plot shows the average reach-and-grasp performance gains for animals receiving physical training and intraspinal delivery of vehicle/quipazine. Inset shows the performance gains as a function of injury severity for individual rats in the two intervention groups. (B) Bar plots show intervention-mediated performance gains after a moderate *vs.* severe SCI. Severely-injured animals are shown in the pink-shaded regions, while moderately-injured rats are shown in the unshaded region of the plots in inset and (B). Numbers (in bold) above the bars quantify the relative performance of rats that received the combined intervention over the physical-training-alone group. Error bars represent standard error of the mean. *p* values were obtained using paired-samples *t* tests. Number of rats included in each group (indicated by 'N'), along with the average injury severity of the group, are reported in parentheses below the bars.

We also plotted therapeutic gains as a function of the injury severity (quantified by the pre-therapy/week 7 performance scores) for individual rats (Figure 2A, inset). Next, we classified the rats according to their injury severities: severely-injured rats, whose functional injury level (or pre-therapy performance) was ≤ 30% (shown in the pink-shaded region), and moderately-injured rats whose pre-therapy performance was greater than 30% (shown in the unshaded region in the inset). The average functional injury level in the moderately-injured cohort was 58% and 59% for rats that received vehicle and quipazine, respectively. We found that there were no significant differences in intervention-mediated reach-and-grasp performance gains in the moderately-injured animals ($t(4) = 0.413$, $p > .05$, paired-samples *t* test; Figure 2B, unshaded region). Delivery of quipazine, therefore, did not produce any added benefit after moderate SCI. On the other hand, the average functional injury level in the severely-injured cohort was 19% and 14% for rats that received vehicle and quipazine, respectively. In stark contrast, the average reach-and-grasp performance gain was 4.3-fold larger in severely-injured rats that received physical training and quipazine compared to those that received physical training alone ($t(4) = 8.066$, $p =$

.001, paired-samples *t* test; effect size = 6.05, very large effect; Figure 2B, pink-shaded region). These results indicate that intraspinal quipazine significantly augments forelimb-motor gains after severe cervical SCI.

Intraspinal quipazine enhances recovery of skilled reach and grasp movements after severe cervical SCI. The forelimb-motor behavior—that serves as targeted physical rehabilitation—consists of two functional movements that are differentially affected after a cervical SCI: reach and grasp (45, 46). To determine the effect of quipazine on the recovery of these movements, we compared intervention-mediated changes in the reach and grasp motor performance, assessed individually, using the data from injured rats reported in Figure 1B.

Figure 3A shows the average weekly reach performance of injured rats as a percentage of their individual pre-SCI maximum scores. The pre-therapy scores of the two groups were similar (57% ± 10% and 53% ± 11% for rats receiving vehicle and quipazine, respectively). Starting with the first week of the intervention, rats that received physical training and quipazine showed greater improvement in their reach performance relative to those that received physical training alone, and this difference continued to widen as therapy progressed. Importantly, performance variability decreased over time in the physical-training-and-quipazine group, with all rats recovering 100% of their pre-injury reach ability at the end of the post-therapy follow-up. In comparison, recovery of reach in the impairment-matched rats receiving physical training alone ranged from 46% to 100%.

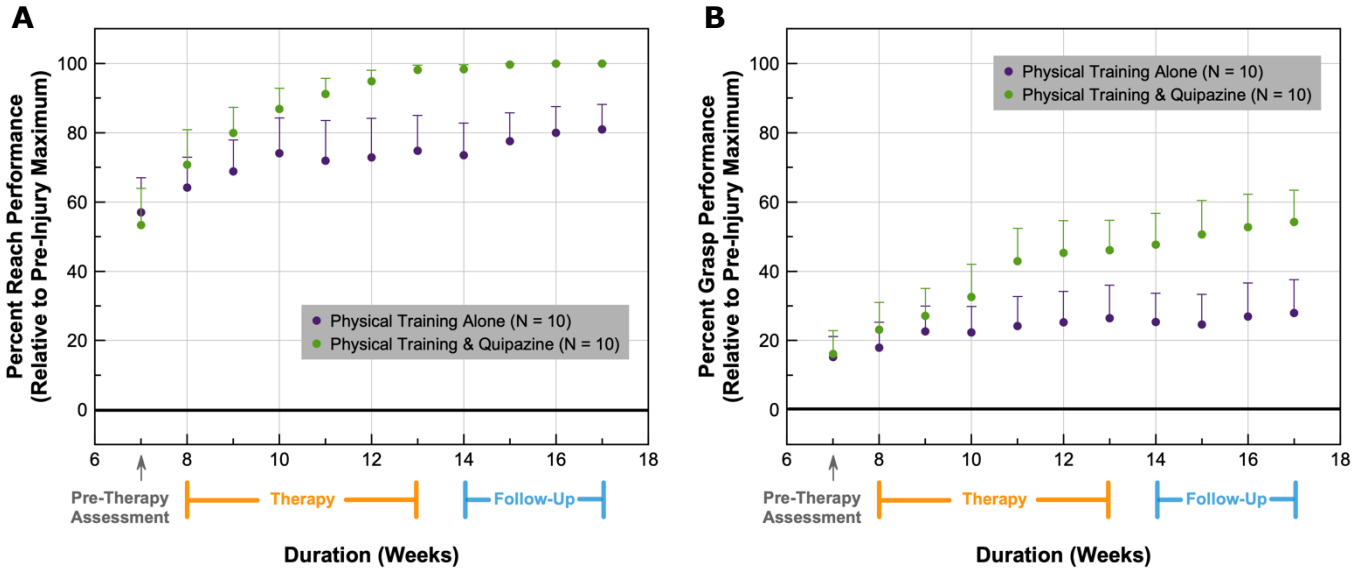

Figure 3. Intraspinal quipazine improves recovery of skilled reach and grasp movements after chronic cervical SCI. (A, B) The average weekly reach (A) and grasp (B) performance of rats, reported as a percentage of their individual pre-SCI maximum scores, is plotted during the pre-therapy, therapy and follow-up periods. Rats received physical training and intraspinal delivery of vehicle/quipazine during the six-week therapy period. Error bars represent standard error of the mean.

In comparison to deficits in reach, grasping ability was more impaired after the cervical contusion SCI with rats starting at appreciably lower pre-therapy (grasp) baselines: 15% ± 6% and 16% ± 7% for rats receiving vehicle and quipazine, respectively (Figure 3B). Additionally, even though trends in motor performance were similar (*cf.* Figures 3A

and 3B), gains were smaller, with rats recovering 54% of their pre-injury grasp ability at the end of the follow-up period in the presence of quipazine and only 28% with physical training alone. Finally, in contrast to changes in reach performance, variability in recovery of grasp continued to remain high during the therapy and follow-up periods and was comparable between the two intervention groups.

Figure 4 shows the average therapeutic gains in reach and grasp produced by our interventions. The average gain in reaching ability was 2.2-fold larger in injured rats that received physical training and quipazine compared to those that received physical training alone ($t(9) = 2.642$, $p = .03$, paired-samples t test; effect size = 0.96, large effect). Quipazine-induced gains in grasp were even larger with injured rats recovering 3.1-fold more grasp compared to those that received physical training alone ($t(9) = 2.622$, $p = .03$, paired-samples t test; effect size = 1.07, large effect). These results indicate that intraspinal quipazine significantly improves recovery of skilled reach and grasp movements after chronic cervical SCI.

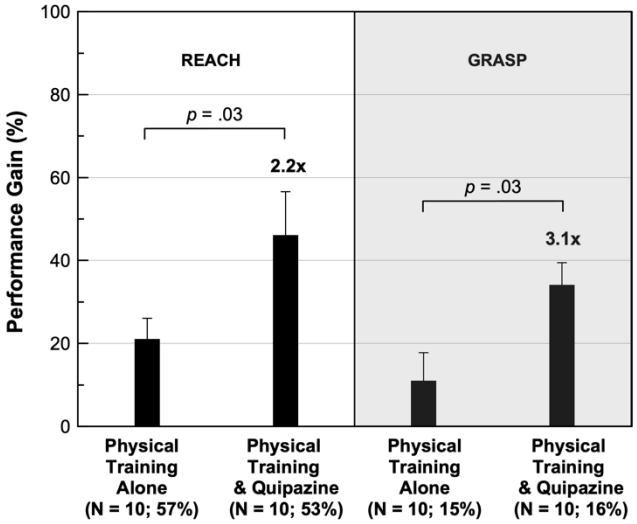

Figure 4. Intraspinal quipazine promotes greater recovery of skilled movements after chronic SCI. The reach (left unshaded panel) and grasp (right gray panel) performance gains are plotted for injured rats receiving physical training and intraspinal delivery of vehicle/quipazine. Numbers (in bold) above the bars quantify the relative performance of rats that received the combined intervention over the physical-training-alone group. Error bars represent standard error of the mean. p values were obtained using paired-samples t tests. Number of rats included in each group (indicated by 'N'), along with the average injury severity (or the pre-therapy reach/grasp baseline) of the group, are reported in parentheses below the bars.

Finally, we plotted the performance gains in reach and grasp as a function of injury severity, using the injury classification developed in Figure 2A (inset), to characterize the effect of our interventions after a moderate *vs.* severe SCI. Using paired-samples t tests, we found that there was no significant differences in the reach or grasp gains produced by our interventions in the moderately-injured animals ($t(4) = -0.320$, $p > .05$ and $t(4) = 0.784$, $p > .05$ for gains in reach and grasp, respectively; Figure 5A). In striking contrast, delivery of quipazine in the spinal cord produced substantially larger improvements in both the reach and grasp ability after severe cervical SCI (Figure 5B). Severely-injured rats that received quipazine showed a 3.1-fold larger gain in their reach ability relative to the physical-training-alone group ($t(4) = 8.139$, $p = .001$, paired-samples t test; effect size = 4.01, very large effect). Quipazine-mediated gains in grasp after severe SCI were also larger

($t(4) = 3.523$, $p = .02$, paired-samples t test; effect size = 10.89, very large effect). On average, rats did not recover any grasp in the absence of quipazine after severe SCI. In the presence of quipazine, the average gain in grasp in the severely-injured animals was 36%. These results demonstrate the effectiveness of intraspinal quipazine in promoting recovery of skilled reach and grasp movements after severe cervical SCI.

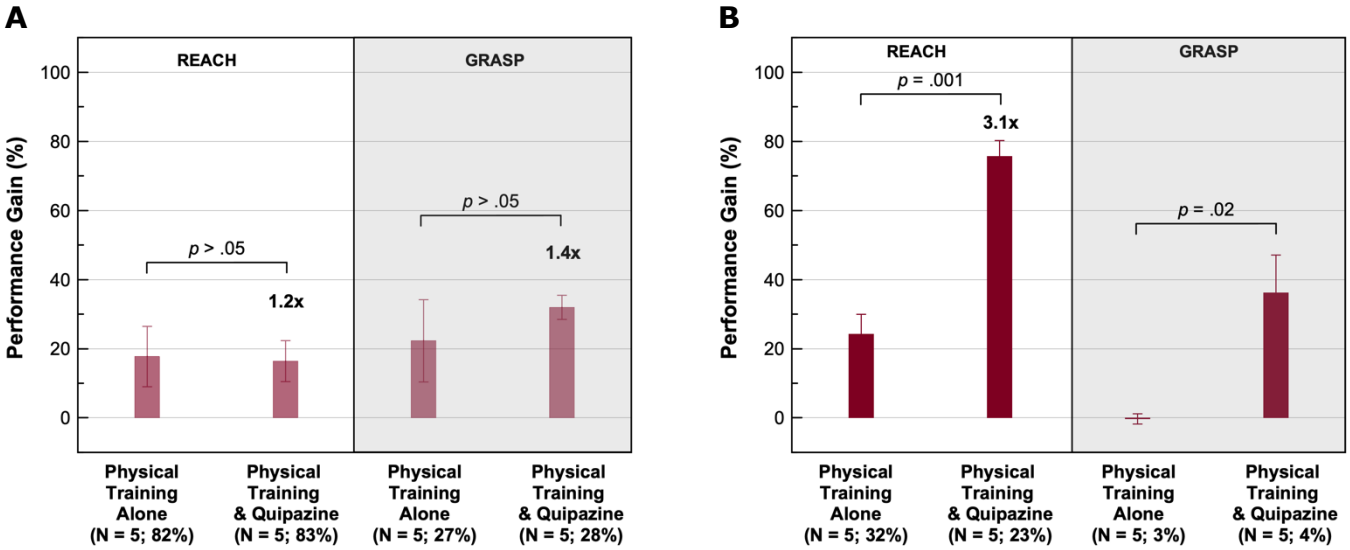

Figure 5. Intervention-mediated forelimb-performance gains after a moderate *vs.* severe SCI. (A, B) The reach (left panel) and grasp (right panel) performance gains after a moderate (A) *vs.* severe (B) SCI are plotted for rats receiving physical training and intraspinal delivery of vehicle/quipazine. Numbers (in bold) above the bars quantify the relative performance of rats that received the combined intervention over the physical-training-alone group. Error bars represent standard error of the mean. p values were obtained using paired-samples t tests. Number of rats included in each group (indicated by 'N'), along with the average injury severity (or the pre-therapy reach/grasp baseline) of the group, are reported in parentheses below the bars. Note that relative gains are not reported for grasp in the severely-injured cohort (B, right panel) due to the lack of gains in the physical-training-alone group (which produced an inflated ratio when comparing gains between the two interventions).

Discussion

This study describes a neuromodulatory intervention that relies on intraspinal delivery of serotonergic agonists to promote forelimb-motor recovery after moderate-to-severe chronic cervical SCI. Chronic motor deficits are one of the most debilitating symptoms experienced by individuals with SCI, and restoration of upper-extremity function has been identified as an important recovery priority (51). Impairments in upper-extremity function can compound difficulties in other areas—such as bowel and bladder management, both of which have also been identified as recovery priorities (51)—to further reduce independence and quality of life. Physical rehabilitation and use-dependent movement interventions are currently the gold standard of treatment after SCI, but they offer limited benefits, especially after chronic trauma (9-14). Our results demonstrate that targeted delivery of quipazine (which is a broad-spectrum serotonergic-receptor agonist; 42), caudal to the lesion, substantially increases the effectiveness of physical rehabilitation in promoting motor recovery in a rodent model of traumatic cervical SCI. Rats in our study received physical rehabilitation combined with intraspinal delivery of quipazine/vehicle. All rats that received quipazine recovered 100% of their pre-injury reach ability and showed substantial improvements in grasp, which led to sizable gains in forelimb-motor performance and reduced post-therapy performance variability in this intervention group. In comparison, physical rehabilitation alone provided significantly reduced benefits, which were more variable across subjects. This difference may be attributed to the beneficial effect of quipazine on motor recovery after severe SCI. While therapeutic benefits in skilled reach and grasp movements were comparable between the two intervention groups in moderately-injured animals, quipazine significantly augmented forelimb-motor gains after severe SCI. Gains in reach were substantially smaller in the absence of quipazine after severe SCI, and physical training alone was completely ineffective in the rescue of skilled grasping movements in severely-injured animals. Notably, our experiments were conducted in a chronic model of cervical SCI. Our data, therefore, highlight an important role for serotonin in restoration of forelimb-motor function after chronic cervical SCI. Lastly, quipazine-mediated improvements in forelimb performance outlasted neuromodulator delivery, suggesting that our intervention induces plasticity in spared spinal circuitry (in contrast to producing acute changes) that drives lasting motor recovery.

Long-lasting changes induced by quipazine and physical training. Quipazine improves motor outcomes by modulating the activity of the serotonergic system in the spinal cord, which undergoes dynamic changes in the setting of SCI. Studies on the activity of 5-HT receptors after SCI have shown upregulation of 5-HT1A, 5-HT2A and 5-HT2C receptors caudal to the lesion, leading to hypersensitivity of motor neurons to serotonergic stimulation (52-56). These receptors remain constitutively active to compensate for depletion of serotonergic input and degeneration of 5-HT neurons (32, 57-59). A previous study of neuromodulator delivery showed that 5-HT pharmacotherapy provided receptor input that reversed 5-HT receptor upregulation following injury (60), suggesting a possible mechanism for the motor improvements produced by quipazine.

However, it is not clear whether the action of 5-HT pharmacotherapy on hyperactive 5-HT receptors produces acute or long-lasting changes in function. In one study, Antri et. al found that subdural injection of quipazine and 8-OHDPAT (agonist for 5-HT1A and 5-HT7 receptors) produced long-term improvements in locomotion up to 75 days after neuromodulator delivery, possibly by reorganization of synaptic networks (61). Additionally, there is some evidence for dendritic plasticity with delivery of serotonergic agents that only

occurs in combination with physical exercise (60), possibly leading to long-term changes in synaptic connections.

In this study, the quipazine and physical training intervention produced markedly increased benefits over physical training alone (62). The gains produced by quipazine may be independent of physical training, or the two interventions may have acted synergistically to produce motor improvements over physical training alone. The role of physical training in SCI recovery has been well-established (63)(reviewed in (64)). Hindlimb training in conjunction with serotonin agonists has been shown to produce improvements in motor recovery (65, 66). The modulation of serotonergic receptors by quipazine, in the setting of exercise induced synapse growth and axonal sprouting (67), may have produced the long-lasting changes in motor function seen with our intervention. However, these previous experiments were performed in a subacute, rather than chronic, SCI recovery phase. Further studies into the duration and molecular changes induced by this intervention are needed to provide more insight into the mechanism of these effects in a chronic SCI model.

Reach vs. grasp training in SCI recovery. We used a reach-and-grasp task as a measure of overall motor performance and analyzed reach vs. grasp as two distinct movements. The use of reach-and-grasp forelimb tasks have been shown to improve task-specific functional recovery after SCI (67) and promote recovery by enhancing plasticity (68). Our study is the first to examine the effect of quipazine on forelimb motor performance using a reach-and-grasp task, as other studies use locomotion as an indicator of motor recovery (65, 69, 70). Given that arm and hand function is associated with increased functional independence in patients with SCI (reviewed in (71)), future studies using forelimb reach-and-grasp tasks are warranted.

However, the reach-and-grasp task quantifies the outcome of successful reach and grasp movements (successes, drops, touches, misses), but does not provide direct information about the movements themselves, such as grip strength, formation of movement, etc. Reaching patterns after SCI are similar to uninjured rats (arm extension and pronation with the paw open) while grasping movements are adaptive (pellet drawn across the surface of shelf rather than flexing digits around pellet (72). Additionally, preparation for grasping, which is indicated by wrist extension during the grasping phase, is usually not seen in SCI (Reviewed in (73)). These differences in movement strategy are not captured in a quantitative outcome of success on the reach-and-grasp task. Using multiple tasks to assess other modalities of recovery could help to further understand the effect of quipazine on motor recovery.

Additionally, the reach-and-grasp task is designed such that attempting a grasping movement is dependent on achieving a successful reach for the pellet. Rats who may have improved in grasping ability independent of reaching were not able to reflect this in their scores. The development of a task that is able to assess these two functional movements independently would more accurately be able to determine the effect of our intervention on these two forelimb behaviors.

Effect of injury severity on motor recovery. Most notable in our results was that severely injured rats showed greater improvements in the reach-and-grasp task with the quipazine intervention compared to moderately injured rats. In comparison to previous studies in our lab using targeted activity-dependent spinal stimulation (TADSS) to activated spared motor pathways (50), quipazine produced similar therapy gains in motor performance (quipazine= 3.39-fold gain, TADSS= 2.89-fold gain), however the cohort of rats used in the present study were more severely injured. The number of successes

achieved by injured rats (see Materials and Methods, Reach-and-grasp scoring) prior to intervention was 3.59 in the TADSS cohort and 0.76 in the quipazine cohort, indicating that TADSS rats started a higher baseline motor performance, but achieved comparable gains to rats receiving quipazine.

Our intervention delivers quipazine caudal to the spinal lesion, where upregulation of 5-HT receptors has been found to occur only in severe contusions (54, 57, 60, 74, 75). Hayashi et. al proposed that there is a serotonergic fiber denervation threshold that is required to produce compensatory upregulation of 5-HT2C receptors (74). More severely injured rats may cross this threshold and be more amenable to the reversal of upregulation of these receptors caused by serotonin agonists (60).

Full fiber degeneration caudal to the lesion is not complete early after SCI (59, 75), indicating that a chronic SCI model, such as the one used in our study, more optimally targets 5-HT receptors at a time point where the denervation threshold required for 5-HT receptor upregulation is reached.

Though normalization of 5-HT receptor levels has previously been seen with delivery of 5-HT pharmacotherapy, this did not translate to improvements in motor function. Ganzer et. al showed that selective serotonin agonists to the 5-HT2C and 5-HT1A receptors were not effective in improving locomotion, but a serotonin precursor (L-5-HTP), which activates all serotonin receptors, did improve locomotion in moderately, and to a lesser degree severely, injured rats (60). Perhaps quipazine, a nonselective serotonin agonist, is able to modulate the appropriate 5-HT receptor targets, causing downregulation of these receptors. These results may guide future studies regarding modulation of the serotonin system to improve motor outcomes.

Conclusions

Our results provide evidence for quipazine delivery in combination with physical training to produce long-lasting improvements in motor performance, which has significant implications for therapeutic delivery of chemical neuromodulators in chronic SCI. These experiments also provide further insight into quipazine's effect on the serotonergic system after SCI. Our findings are supported by previous studies regarding receptor upregulation after SCI (52, 54), and provide new insight into these effects in a model of chronic SCI with long-lasting therapeutic improvements. Further investigation is necessary to study the reorganization of 5-HT receptors after SCI and quipazine's mechanism of action in improving motor performance. Future studies will address combination neuromodulator therapies, such as combined BDNF and quipazine therapy, to enhance plasticity after injury via multiple mechanisms.

Materials and Methods

All animal-handling, training and surgical procedures were approved by the University of Washington's Institutional Animal Care and Use Committee, and they conformed to the National Institutes of Health's Guide for the Care and Use of Laboratory Animals.

Subjects. Experiments were performed with 20 adult female Long-Evans rats with average pre-injury weight 306 grams (range: 266 g - 354 g).

Behavioral training. Rats were trained on a forelimb reach-and-grasp task (44) during their dark (i.e., active) cycle, using operant-conditioning protocols (47) for food-pellet rewards. To initiate training, animals were placed in a clear Plexiglas arena with two vertical slits near the lower-front corners. Food pellets were inserted inside small dimples on a plastic block placed outside the arena in front of the vertical slits. A gap between the behavioral-training arena and the plastic block ensured that food pellets would fall and land out of reach when not firmly grasped by the rats. After identification of forelimb dominance, rats were trained to reach out through the slit, grasp the food pellet placed on the block with their dominant forepaw, and bring it through the slit to their mouths. The width and position of the slit were chosen to ensure that rats can reach the pellets with only one forelimb; after SCI, this design discourages reaching with the uninjured forelimb, thus focusing training on the impaired limb. Rats were trained to perform the reach-and-grasp task until they reached a reach-and-grasp score of 80% or higher (which took 1 - 4 weeks).

Reach-and-grasp scoring. Each physical training session consisted of 20 trials with a food pellet, with a short unscored warmup of up to 5 trials. Performance on the session was quantified based on a cumulative total of the outcome of each individual pellet retrieval. There were five possible outcomes for each pellet retrieval attempt:
1. Success: the rat retrieved the food pellet and brought it to its mouth with the injured forelimb
2. Drop-inside: the rat retrieved the food pellet, but dropped the pellet inside the arena before bringing it to its mouth
3. Drop-outside: the rat retrieved the food pellet, but dropped the pellet outside the arena
4. Touch: the rat was able to extend the forelimb to touch the pellet, but was not able to grasp the pellet
5. Miss: the rat was not able to touch the pellet

These outcomes: success, drop-inside, drop-outside, touch, and miss, were weighted into a final score based on the individual reach and grasp components of each outcome. The reach score assigns 0.5 for success, drop-inside, drop-outside, and touch. These are assigned equal weights due to the similar level of forelimb reaching ability required for each outcome. The grasp score assigns 0.5 for success, 0.33 for drop-inside, and 0.17 for drop-outside. These weights were distributed based on the respective level of grasp ability required for each outcome. Trials resulting in misses were assigned a value of zero. The sum of 20 individual trials were added for each total scoring component (reach score, grasp score, total reach-and-grasp score).

Table 1. Reach and grasp scoring per trial.

	Success	Drop-inside	Drop-outside	Touch	Miss
Reach score	0.5	0.5	0.5	0.5	0
Grasp score	0.5	0.33	0.17	0	0
Reach-and-grasp score	1	0.83	0.67	0.5	0

Spinal cord injury. Rats that achieved a reach-and-grasp score of 80% or higher received a unilateral moderate-to-severe dorsal spinal-contusion injury at the border of C4-C5 segments on their dominant forelimb side. Rats were anesthetized to a surgical plane using ketamine/xylazine and verified by toe pinch and ocular blink reflex. They were given antibiotics (Baytril), a local intradermal anesthetic (lidocaine and bupivacaine) along the incision line and scrubbed into a sterile field. A dorsal approach to the cervical spinal column was performed, followed by a hemilaminectomy of vertebral C4 of the rat's trained forelimb. Spinal clamps of the Ohio State impactor (OSI) were then placed on the lateral processes of C3 and C5, fixing the spinal column into a suspended position. The OSI probe was aligned the dorsal root entry zone of the expose cord, lowered until probe sensors detected contact, then the OSI was triggered to rapidly displace the cord to simulate an impact injury. After injury, the rat was removed from the OSI, the spinal contusion was evaluated, covered in saline-soaked gel foam, and the approach was sutured closed. Rats recovered with fluid and heat support until awake. All received post-op meds (Baytril 0.2mg/mL in drinking water, SR buprenorphine 1mg/kg) and had bladders expressed twice daily until full bladder function returned. All surgical procedures were done using sterile techniques.

Cannula Implant. Approximately four weeks post-injury, rats were implanted with an intraspinal cannula in the ipsilesional C7 segment. The custom-made cannula consisted of a 3mm length of 0.13mm lumen polyurethane tubing (Instech Laboratories Inc), of which 2mm was inserted into the end of a length of 0.3mm lumen silicon tubing (Dupont), then secured with 4-0 silk suture (AD surgical) and liquid silicon medical adhesive (Factor II, Inc). A polyamide disc was placed at the tubing junction to act as a depth stop, limiting the polyamide tubing depth to 1mm.
Rats were anesthetized (isoflurane, SomnoSuite), placed on a heating pad, and given antibiotics (Baytril, 0.7mg/kg), corticosteroid (dexamethasone, 0.5mg/kg), analgesic (meloxicam, 1mg/kg) and fluid support (Lactated Ringer's solution). Surgical plane was verified via toe pinch and ocular reflex, and vitals were continually monitored via SomnoSuite. After establishing surgical sterility and a sterile field, an incision was made along the dorsal midline above C7, the muscle was separated and retracted, and the spinal column was exposed. A C7 and partial C6 hemilaminectomy was on the ipsilesional vertebrae. The cannula was flushed with 70% isopropanol followed by sterile saline, then was primed with saline, after which the pump was tied off with 4-0 silk suture (AD Surgical). The dura over the C7 dorsal root entry zone was incised and the polyurethane cannula was inserted 1mm into the cord, as set by the depth stop. The dura was closed atop the depth stop and around the cannula with 7-0 silk suture, anchoring it in place. The remaining tubing extended dorsally through the muscle, which was closed with 4-0 PGA suture (AD Surgical), and coiled into a subdermal pocket, which was closed with 5-0 nylon suture (AD Surgical). Animals recovered on a heating pad, given post-op analgesic (SR buprenorphine, 1mg/kg) and monitored until waking, after which they returned to their home cage and had antibiotics added to their water (Baytril 100, 0.2mg/mL).

Pump Implant. About 2 weeks after receiving the cannula, rats were anesthetized with isoflurane, given antibiotics (baytril, 0.7mg/kg), analgesics (meloxicam, 1mg/kg), and fluid support (Lactated Ringer's Solution). A small incision was made above the cannula implant, exposing the pump end of the tubing, from which 5mm were removed. A second incision was made several centimeters rostrally and a subdermal pocket was created for a pre-programmed battery-operated microinfusion pump (Alzet, iPrecio SMP-200, (49)) via blunt

dissection. The pump tubing was trimmed to 2cm, and the pump was primed with sterile saline. Then the cannula tubing was tunneled subdermally to the pump site, where it was attached to the pump tubing using 4-0 silk suture and liquid silicon (Qwik Sil). The pump was anchored inside the pocket with silk suture and both incision sites were closed with 5-0 nylon. Animals recovered on a heating pad and returned to their home cages.

Saline was delivered during two weeks of post-operative recovery, followed by one week of pre-therapy reach-and-grasp assessment on the pellet-retrieval task (also in the presence of saline). Before the start of therapy, fluid in the pump reservoir was exchanged from saline to neuromodulators (via a percutaneously accessible septum). A saline-to-saline exchange was performed on animals receiving vehicle.

Injury-matching and group selection. To reduce bias for the estimated treatment effect, we used a matched comparison design to assign rats to the treatment and control groups based on the severity of injury produced. Table 1 describes the five groups to which rats were assigned based on injury level, which was determined by the pre-therapy reach-and-grasp performance (See data analysis, Materials and Methods).

Table 2. Injury-matched groups.

Injury-matched group	Pre-therapy performance (min)	Pre-therapy performance (max)	Range	Physical Training only rats (n)	Physical Training & Quipazine rats (n)
1	70.37	78.04	7.67	2	2
2	55.89	66.76	10.87	1	1
3	35.68	47.54	11.86	2	2
4	16.14	30.01	13.87	3	3
5	6.70	11.23	4.54	2	2
Total	6.70	78.04	71.34	10	10

To analyze the effect of quipazine based on SCI severity, rats with a pre-therapy performance of ≤ 30% were considered severely injured (Injury-matched groups 4 and 5), and rats with a pre-therapy performance of >30% were considered moderately injured (injury matched groups 1-3).

Physical training and neuromodulator/vehicle delivery. Therapy, consisting of physical rehabilitation and neuromodulator/vehicle delivery, was administered during weeks 8 – 13 post injury. This delay between injury and start-of-therapy was deliberately chosen to allow the injury to translate to a chronic SCI model (50). Physical rehabilitation consisted of ten sessions per week of training of the impaired forelimb on the pellet-retrieval task, and occurred during periods of ongoing neuromodulator/vehicle delivery. All rats in all therapy groups received the same amount of physical training per week. To eliminate biases in scoring, all training was performed by researchers blinded to the therapy group of the rats.

Neuromodulator/vehicle (saline) was administered intraspinally for 4.5 hours/day. Rats received quipazine or vehicle at a rate of 20.0 μg/week during the therapy period (76, 77), with the exception of two rats in the control group who received no fluid due to cannula clogging observed during the terminal surgery.

Pumps were refilled with quipazine/saline as needed during therapy and post-therapy periods. Rats were briefly sedated using isoflurane for biweekly reservoir-volume checks on the pumps to confirm fluid delivery and rule out clogging issues.

After completion of therapy, rats were tested in the pellet-retrieval task for an additional four weeks (in the presence of saline) to assess if therapeutic gains were retained in the absence of neuromodulators. All rats in all groups received (re-)training on pellet retrieval during pre-therapy assessment, therapy and follow-up periods.

Terminal analyses. After completion of post-therapy follow-up, all rats from all therapy groups underwent a terminal surgery during which the integrity of all fluidic junctions, flow through the entire line, location and entrance angle of the cannula into the spinal cord, and the location of the (cortical and spinal) microwires were evaluated to confirm correct placement throughout the experimental timeline.

Data analyses. During the training period, rats received 10 training sessions per week (See physical training/neuromodulator delivery). The Pre-SCI maximum score was the highest weekly average score obtained during the training period.
To assess motor recovery, the weekly percent-reach-and-grasp performance after SCI was expressed as a percentage of the rat's individual pre-SCI maximum:

$$\text{Reach-and-grasp performance (\%)} = \frac{\text{weekly average reach-and-grasp score}}{\text{pre-SCI maximum score}} \times 100$$

Similarly, to evaluate reach vs. grasp as individual functional movements, we expressed the reach or grasp score as a percentage of the rat's individual pre-SCI maximum.

$$\text{Reach performance (\%)} = \frac{\text{weekly average reach score}}{\text{pre-SCI maximum reach score}} \times 100$$

$$\text{Grasp performance (\%)} = \frac{\text{weekly average grasp score}}{\text{pre-SCI maximum grasp score}} \times 100$$

To account for differences in the average injury level at week 7 across groups, we calculated the therapeutic performance gains. Weekly performance gain represented the improvement in motor performance after therapy:

Performance gain (%) = average post-therapy performance (%) – average pre-therapy performance (%)

Statistics. All weekly performance values are reported as mean ± standard error.
Paired samples t-test was used for performance comparisons between the PTQ and PT groups. Before running paired t-tests, we verified that the performance differences followed a normal distribution. Normality was assessed using the Shapiro-Wilk test. Wilcoxon signed-rank test, which is the nonparametric equivalent of the paired t-test, was used when the data were not normally distributed. All statistical analyses were performed in IBM SPSS Statistics (version 27).
In addition to assessing statistical significance, we also quantified the magnitude of the effect using the 'effect size' metric, which is the standardized difference of the mean of two groups, according to the following equation (78).

$$\text{Effect size} = \frac{\text{(Mean of Experimental Group-Mean of control group)}}{\text{Population Standard Deviation}}$$

Population standard deviation was estimated by pooling the (sample-size-weighted) standard deviations of the experimental and control groups, using the equation below, when standard deviations of the two groups were similar. Similarity of standard deviations was assessed by calculating the difference in coefficients of variance. Otherwise, the standard deviation of the control group was used. These metrics correspond to Hedges' g (79) and Glass' delta (80) effect-size indices, respectively.

$$\text{Pooled Standard Deviation} = \text{SQRT}\left(\frac{(N_E-1) \times SD_E^2 + (N_C - 1) \times SD_C^2}{(N_E + N_C - 2)}\right)$$

Here, N_E, SD_E, N_C and SD_C are the sample sizes and standard deviations of the experimental and control groups, respectively. Lastly, effect-size descriptors developed by Cohen (78) and extended by Rosenthal (81) which are summarized in Table 2, were used for qualitative interpretation of effect sizes.

Table 3. Effect Size and Interpretation.

Effect Size	**Interpretation**
$0.00 < d < 0.20$	Negligible or Trivial
$0.20 \leq d < 0.50$	Small
$0.50 \leq d < 0.80$	Medium
$0.80 \leq d < 1.30$	Large
$d \geq 1.30$	Very Large

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