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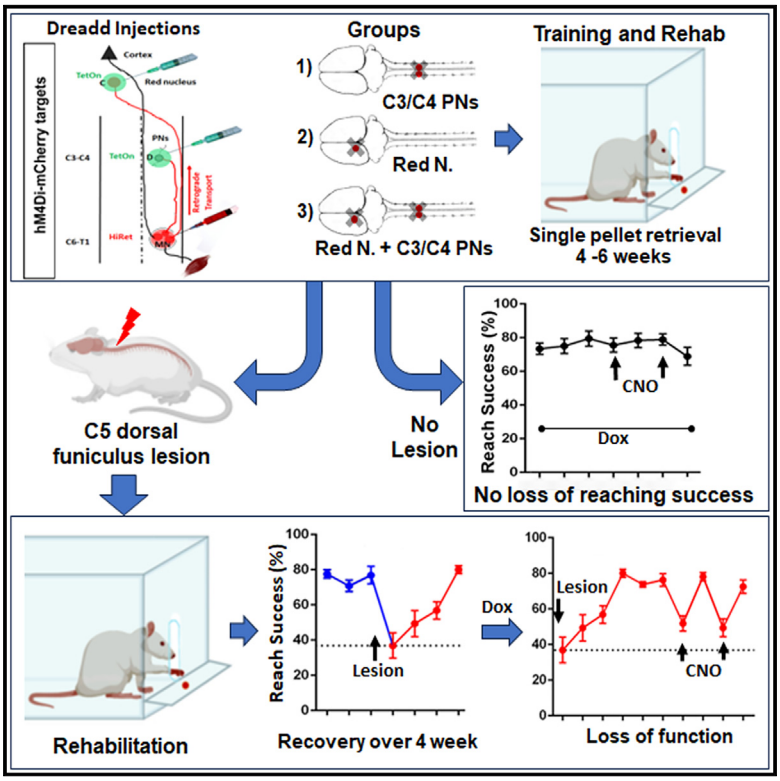
Compensatory adaptation of parallel motor pathways promotes skilled forelimb recovery after spinal cord injury

Graphical abstract



Highlights

- Rehabilitation after dorsal funicular lesions promotes recovery of skilled reaching
- Partial recovery is mediated by either rubrospinal or C3/C4 propriospinal tracts
- Silencing both these tracts eliminates rehabilitation mediated recovery
- Rehabilitation induces CST sprouting into the red nucleus and C3/C4 spinal cord

Authors

Imran S. Sheikh, Kathleen M. Keefe, Noelle A. Sterling, ..., Xiao-Ming Xu, Lynn G. Kirby, George M. Smith

Correspondence

george.smith@temple.edu

In brief

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Compensatory adaptation of parallel motor pathways promotes skilled forelimb recovery after spinal cord injury

Imran S. Sheikh,[1] Kathleen M. Keefe,[1] Noelle A. Sterling,[1] Ian P. Junker,[1] Chen Li,[2] Jie Chen,[1] Xiao-Ming Xu,[3] Lynn G. Kirby,[2] and George M. Smith[1,4,*]

[1]Department of Neuroscience, Shriners Hospitals Pediatric Research Center, Center for Neural Rehabilitation and Repair, Lewis Katz School of Medicine at Temple University, Philadelphia, PA 19140, USA

[2]Department of Anatomy and Cell Biology, Center for Substance Abuse Research, Lewis Katz School of Medicine at Temple University, Philadelphia, PA 19140, USA

[3]Spinal Cord and Brain Injury Research Group, Stark Neurosciences Research Institute, Indiana University School of Medicine, Indianapolis, IN 46202, USA

[4]Lead contact

*Correspondence: george.smith@temple.edu

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SUMMARY

Skilled forelimb patterning is regulated by the corticospinal tract (CST) with support from brainstem regions. When the CST is lesioned, there is a loss of forelimb function; however, if indirect pathways remain intact, rehabilitative training can facilitate recovery. Following spinal cord injury, rehabilitation is thought to enhance the reorganization and plasticity of spared supraspinal-propriospinal circuits, aiding functional recovery. This study focused on the roles of cervical propriospinal interneurons (PNs) and rubrospinal neurons (RNs) in the recovery of reaching and grasping behaviors in rats with bilateral lesions of the CST and dorsal columns at C5. The lesions resulted in a 50% decrease in pellet retrieval, which normalized over four weeks of training. Silencing PNs or RNs after recovery resulted in reduced retrieval success. Notably, silencing both pathways corresponded to greater functional loss, underscoring their parallel contributions to recovery, alongside evidence of CST fiber sprouting in the spinal cord and red nucleus.

INTRODUCTION

Cervical injury to the spinal cord results in diminished or complete loss of skilled forelimb mobility, depending on the severity of the injury.[1] Such goal-directed reaching and grasping behaviors are primarily directed by corticospinal neurons. Corticospinal axons from forelimb motor regions preferentially synapse within the cervical spinal cord with only minor projections within the brainstem. Ablation of these neurons results in major deficits in forelimb function, particularly targeted reaching, grasping, and retrieval of food pellets.[2–4] Likewise, selective lesions of the CST results in diminished goal-directed or skilled reaching, and although axonal regeneration does not spontaneously occur, such lesions are often associated with functional recovery, particularly after rehabilitative training.[5–8] Under such conditions, restoration of task-specific motor control must be relayed past the lesion though supplemental pathways to induce sufficient excitation of appropriate spinal motor pools.[1,7] After pyramidotomy, which completely lesions the CST, partial recovery is mediated by relaying motor commands through the rubrospinal tract.[9–12] Ablating the red nucleus or cutting the rubrospinal tract in combination with pyramidotomy abolished recovery.[13,14] Likewise, dorsal funiculus lesions within the mid-cervical region of the spinal cord, which lesion 95% of CST axons, also result in diminished forelimb function, showing almost complete recovery after 4–6 weeks of rehabilitative training.[5,7,8,15]

Multiple studies focusing on recovery of locomotion after incomplete spinal cord injuries conclude plasticity of intraspinal circuits establishes detours bypassing the lesion.[16–20] Such alternative circuits are composed of endogenous propriospinal networks modified by task-oriented rehabilitation. Propriospinal neurons localized throughout the spinal cord often traverse multiple spinal cord segments to provide intersegmental connectivity, enabling adaptive reorganization after injury.[11,21,22] Within the C3-C4 region of the spinal cord is a unique type of propriospinal neuron with a bifurcating axon; one branch descends to innervate neurons in the forelimb motor pools within the C6-T1 spinal segments and the other branch ascends to innervate the lateral reticular nucleus (LRN).[23,24] Cervical PNs receive motor input from cortico-, rubro-, reticulo-, and tectospinal tracts[23,24] as well as sensory information from the periphery to modulate limb trajectory during movements.[25,26] Selective lesions interrupting descending motor or ascending sensory transmission indicate the importance of C3-C4 neurons for target-directed reaching behaviors. For example, targeted reaching recovered after dorsolateral spinal cord lesions in a cat, which severed

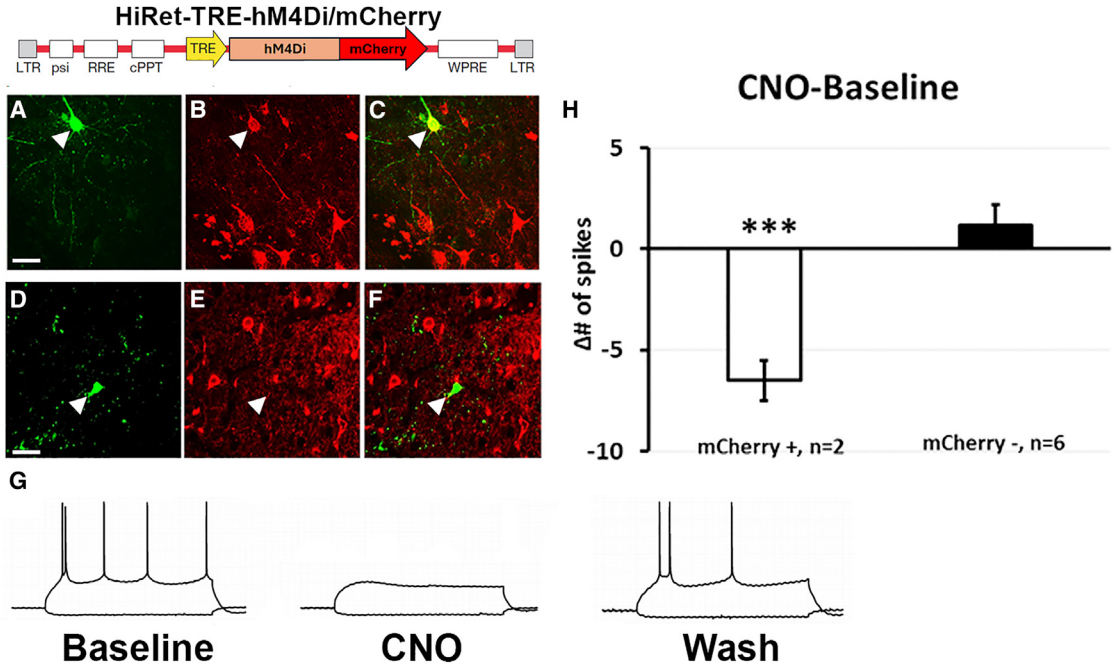

Figure 1. Inducible inhibitory DREADDs attenuate cortical neuron excitability

Fluorescent images of cortical slices with neurons transduced with AAV2-TetOn and HiRet-TRE-hM4Di-mCherry. Example of recorded cortical neuron that expressed hM4Di-mCherry; (A) biocytin, (B) mCherry and (C) merged. Example of recorded cortical neuron (arrowhead) that did not express hM4Di-mCherry; (D) biocytin, (E) mCherry and (F) merged. Recorded neurons showing attenuation upon CNO application in neuronal excitability were hM4Di+/biocytin+. Recorded neurons that showed no CNO-mediated attenuation were hM4Di-/biocytin+.

(G) Change in number of spikes measured during electrical excitability of whole-cell patch clamped cortical neurons expressing hM4Di. Two-tailed unpaired t-test was significant: t(7.529, 6); $p = 0.0003$.

(H) Representative electrophysiology traces from a recorded hM4Di+ cortical neuron. Normal cell firing was observed during baseline measurements. CNO application temporarily attenuated cell firing that returned to normal upon washing out cell with ACSF. Data is mean ± SD. N = 2–6. ***$p < 0.001$. Scale bars in (A) and (D) are 50 μm.

CST and RST input to C6–T1 forelimb MNs.[25] Electrophysiological studies identified the presence of excitatory inputs through C3-C4 PNs thought responsible for the retention of forelimb target-directed reaching.[25,27] In uninjured primates, synaptic silencing of C3-C4 PNs by virally expressed tetanus toxin light chain caused deficits in forelimb grasping behaviors.[28] After spinal cord injury (SCI) in rodents, PNs can sprout and reorganize to promote relays bypassing lesions to re-establish supraspinal input to denervated MNs permitting recovery of locomotion.[17] Previous reports examining supraspinal axon sprouting with different treatment modalities postulate that recovery of function may utilize spared endogenous neuronal networks after injury.[21,29] Although PNs play a significant role in recovery of locomotor behaviors in mammals because they form bypass circuits around spinal cord lesions, it is unclear whether they are responsible for functional recovery of target-directed reaching behaviors.

RESULTS

Silencing of C3-C4 PNs, RSNs or C3-C4 PNs + RSNs does not affect pre-injury reaching behaviors

Reaching and grasping is primarily mediated through activity of the CST but silencing the C3-C4 PNs in monkeys produces a transient reduction in hand dexterity associated with grasping.[28] To determine if C3-C4 PNs are involved in reaching or grasping behaviors of uninjured and injured rats, we generated a High Retrograde transportable lentivirus (HiRet)[30] encoding a Tet-On-inducible hM4Di expression cassette (Figure 1, HiRet-tre-hM4Di-mCherry). This lentivirus serotype uses a chimeric Rabies –G envelope with selective uptake into synapses, showing excellent expression within C3-C4 propriospinal and rubrospinal neurons (RNs) when injected into the C6-T1 spinal cord.[31] To verify neuronal silencing is mediated by this construct, cortical neurons in the forelimb motor area were co-transduced with HiRet-tre-hM4Di-mCherry and AAV2-TetOn and examined for neuronal activity in *ex vivo* brain slice electrophysiology studies (Figure 1). Prior to evaluation of brain slices, rats were treated with doxycycline treatment for 7 days to induce expression of hM4Di-mCherry prior to slice preparation. Imaging showed excellent expression of mCherry within cortical neurons and biocytin staining (green) in patched neurons (Figures 1A–1C), whereas control neurons did not express mCherry (Figures 1D–1F). Whole-cell patch-clamping studies reviled good silencing of cortical neurons in the presence of CNO and return of activity after washout of the drug (Figures 1G and 1H). These studies were also repeated with hippocampal and DRG cultures showing identical results (not shown).

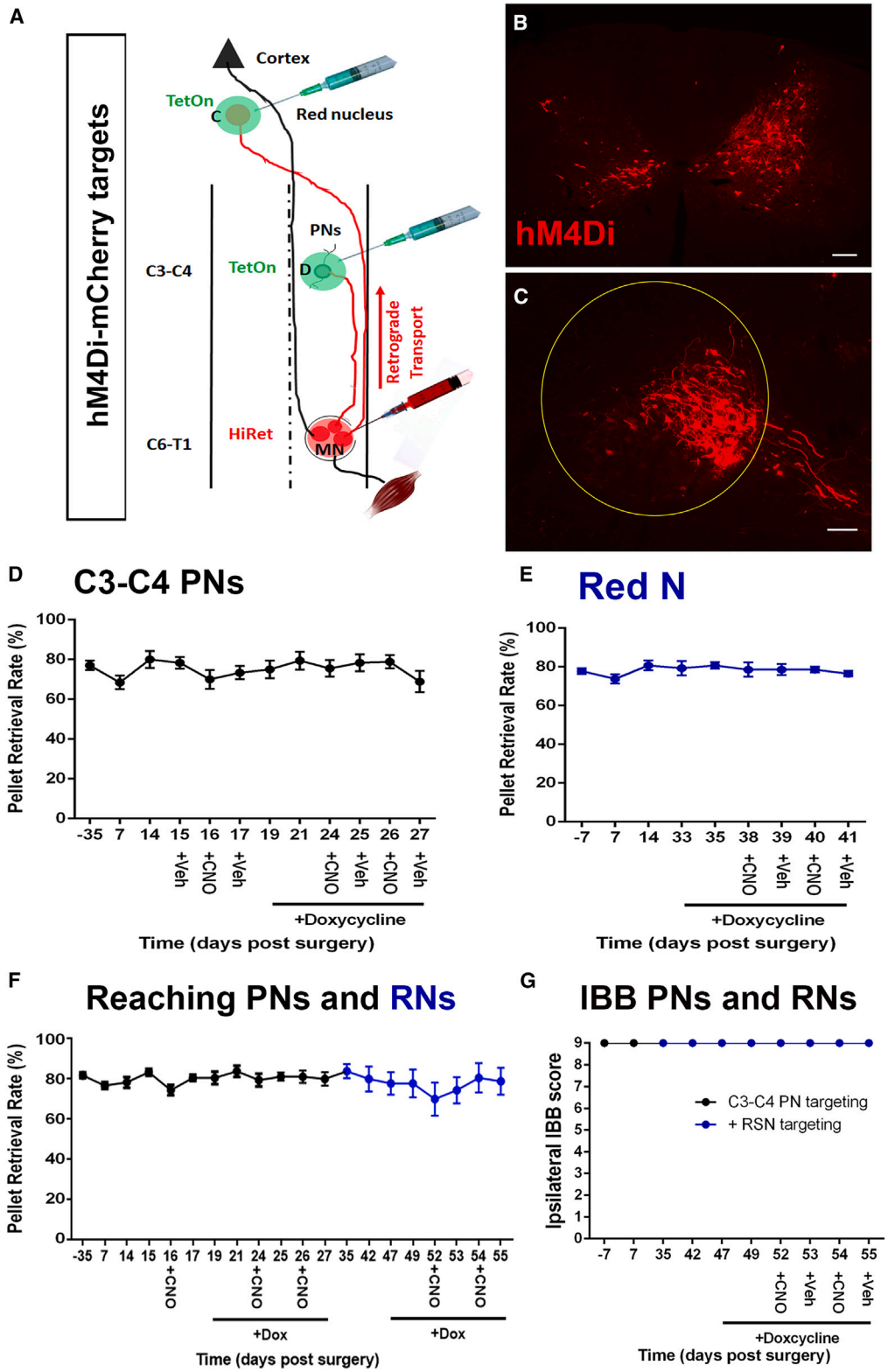

Figure 2. Neuronal silencing of C3-C4 PNs and/or RSNs in normal rats does not affect forelimb reaching behavior

(A) Silencing of C3/C4 PNs or RSNs was mediated by a 2 viral vector system for doxycycline inducible expression of hM4Di. For this study HiRet-tre-hM4Di-mCherry was injected into laminae VII of the C6-T1 spinal cord where it is preferentially taken up at synapses and retrogradely transported to multiple

(legend continued on next page)

First, we wanted to determine if either C3-C4 PNs or RNs participated in reaching or grasping in normal rats. Since these neurons terminate within the forelimb motor pools, we first retrogradely labeled them by injecting HiRet-tre-hM4Di-mCherry within the ventral horn near C6-T1 MNs ipsilateral to the preferred reaching forepaw. These injections were followed by injections of AAV2-TetOn into either (1) C3-C4 region of the spinal cord at the location of PN cell bodies bilaterally, (2) red nucleus contralateral to the preferred forepaw, or (3) both C3-C4 bilaterally and the contralateral red nucleus (Figure 2A). This intersectional genetic method led to specific mCherry expression of propriospinal neurons in the C3/C4 spinal cord (Figure 2B) or RNs (Figure 2C). We previously showed HiRet-GFP injected into the C6-T1 region labels the majority of the C3/C4 PNs (>20,000 bilaterally) and most RNs (>1,000).[31] In this study, we observe very similar densities of hM4Di-mCherry labeled neurons within the PNs and RNs, respectively. Neurons within the C3-C4 spinal cord were primarily localize within laminae IV through VIII ipsilaterally and lamina VII-VIII contralaterally.

To determine if either C3-C4 PNs or RNs have a role in mediating reaching and grasping prior to any injury, we silenced them individually or together. Animals were trained to reach and grasp a food pellet using the single pellet-reaching task. Once trained, forelimb motor scores remained unchanged for several weeks after viral injections when compared to baseline scores. With most all reaches, they can successfully reach thought the opening toward the pellet and grasp the pellet, sometimes after several attempts. To grasp the pellet, they extend their digits and pronate their hand over the top of the pellet. Once they contact the pellet, they close their hand and retract the pellet. Sometimes they drag the pellet off the shelf or eat it with both hands, but mostly they lift the pellet from the shelf and bring it directly to their mouth. CNO was administered (14–16 days post-surgery) in the absence of doxycycline to determine any behavioral effects of clozapine metabolites (Figures 2D and 2F). To induce expression of hM4Di, doxycycline (30 mg/kg, s.c.) was administered twice a day for 9 days and CNO (4 mg/kg) injected on day 5 and 7 after the first doxycycline injection. We observed no deficits in ipsilateral forelimb reaching behavior with silencing of either the C3/C4 PNs (Figure 2D), RNs (Figure 2E), or both simultaneously (Figure 2F) 30–40 min after CNO (4 mg/kg, i.p.) administration to rats. These data show that unlike monkeys, silencing rat C3/C4 PNs does not affect the ability to reach and grasp food pellets. Likewise, we observed no deficits in wrist and hand movements while eating cereal pieces according to the Irvine, Beattie, Bresnahan (IBB)[32,33] forelimb test after CNO injections for all groups (Figure 2G).

C3-C4 PNs or RNs mediate partial recovery of reaching behavior after C5 dorsal funiculus injury

The cortex provides major input for voluntary movements including hand dexterity, digit function and forelimb reaching and grasping[3,34] and as shown in the first study, functions mostly independent of PNs or RNs. Since previous publications show cortical motor neuron ablation or silencing greatly reduced reaching and grasping behavior,[2,4] we proceeded to performing lesion studies. C5 dorsal funiculus lesions sever the dorsal CST containing ~95% of CST axons along with the fasciculus gracilis and cuneatus while sparing the majority of the gray matter and the lateral and ventral funiculi containing the rubrospinal and propriospinal axon tracts, respectively.[3,35] It is well established that after C5 dorsal funiculus injuries, target directed reaching behavior significantly diminishes, but recovers to normal levels over a period of 4 weeks with training. To examine the roles of C3-C4 PNs and RNs involved in the recovery of reaching and grasping, we selectively silence them individually and together following specific timelines (Figure 3A).

In all rats, GFAP staining highlighted the C5 lesion border to ensure the injury did not extend into the lateral or ventral funiculi (Figures 3B and 3C). Since CST axons express PKCγ, upper thoracic sections (Figure 3D) will show an absence of PKCγ staining in the dorsal funiculus, but not the dorsal horn, to verify that the dorsal CST was completely lesioned.[36] Any animals with intact or incomplete dorsal CST lesions, staining positive for PKCγ in the CST, were excluded from the study. For these studies, HiRet-Tre-hM4Di-mCherry was injected into the C6-T1 spinal cord and AAV2-TetOn injected into either the C3-C4 spinal cord (Figure 3E) or red nucleus (Figure 3H). Seven days post injection and before lesioning, reaching and grasping studies showed no deficits, even with expression of hM4Di and CNO treatment, similar to non-lesion rats in Figure 2.

All rats received a C5 dorsal funiculus lesion on day 45 and showed significant single-pellet retrieval errors of ~50% of pre-lesion scores seven days post lesion. These rats show multiple errors, with some reaching attempts they fail to reach through the slit and hit their hand on the bottom part of the shelf. For others, they reach through the slit but show either targeting errors, where they over or underreach the pellet or lack of protonation -trying to hook and swipe the pellet back through the opening. Once they contact the pellet they rarely grasp and lift it, mostly dragging it off the shelf either onto the floor or grabbing it with both forepaws as it is removed from the shelf. Over the subsequent 28 days of rehabilitation, pellet retrieval scores increased to pre-lesion baseline levels, however, they seem to drag the pellet off the shelf slightly more often instead of lifting it. (Figures 3F and 3I). Injection of CNO without doxycycline pretreatment showed no significant change in success of pellet retrieval, again indicating no behavioral deficits by CNO alone. To determine which pathways are involved in recovery, we administered doxycycline for nine days to induce expression of hM4Di. To silence these neuronal populations, CNO was administered twice, once at 5 and again 7 days after the start of doxycycline treatment. Animals expressing hM4Di targeted to the

neuronal populations innervating that region. AAV-TetOn was stereotaxically injected into either the red nucleus, C3/C4 spinal cord or both. Representative images showing labeling of C3 propriospinal neurons (B) or red nucleus (C). Silencing of C3/C4 propriospinal neurons (D), rubrospinal neurons (E), or both (F) in normal rats shows no change in pellet retrieval with the administration of CNO either before or after induction of hM4Di expression with doxycycline. Silencing of both C3/C4 propriospinal neurons and rubrospinal neurons (G) failed to show reductions in hand and digit dexterity before or after CNO administration. Repeated measures one-way ANOVA was found not significant in D) $F(4.921, 39.37) = 2.368$; $p = 0.0578$, $n = 9$; E) $F(3.445, 20.67) = 0.9004$; $p = 0.4695$, $n = 7$; and F) $F(4.166, 29.16) = 2.008$; $p = 0.1172$, $n = 8$ for each group. Data is mean ± SEM. Scale bars in panels B is 250 μm and panel C is 150um.

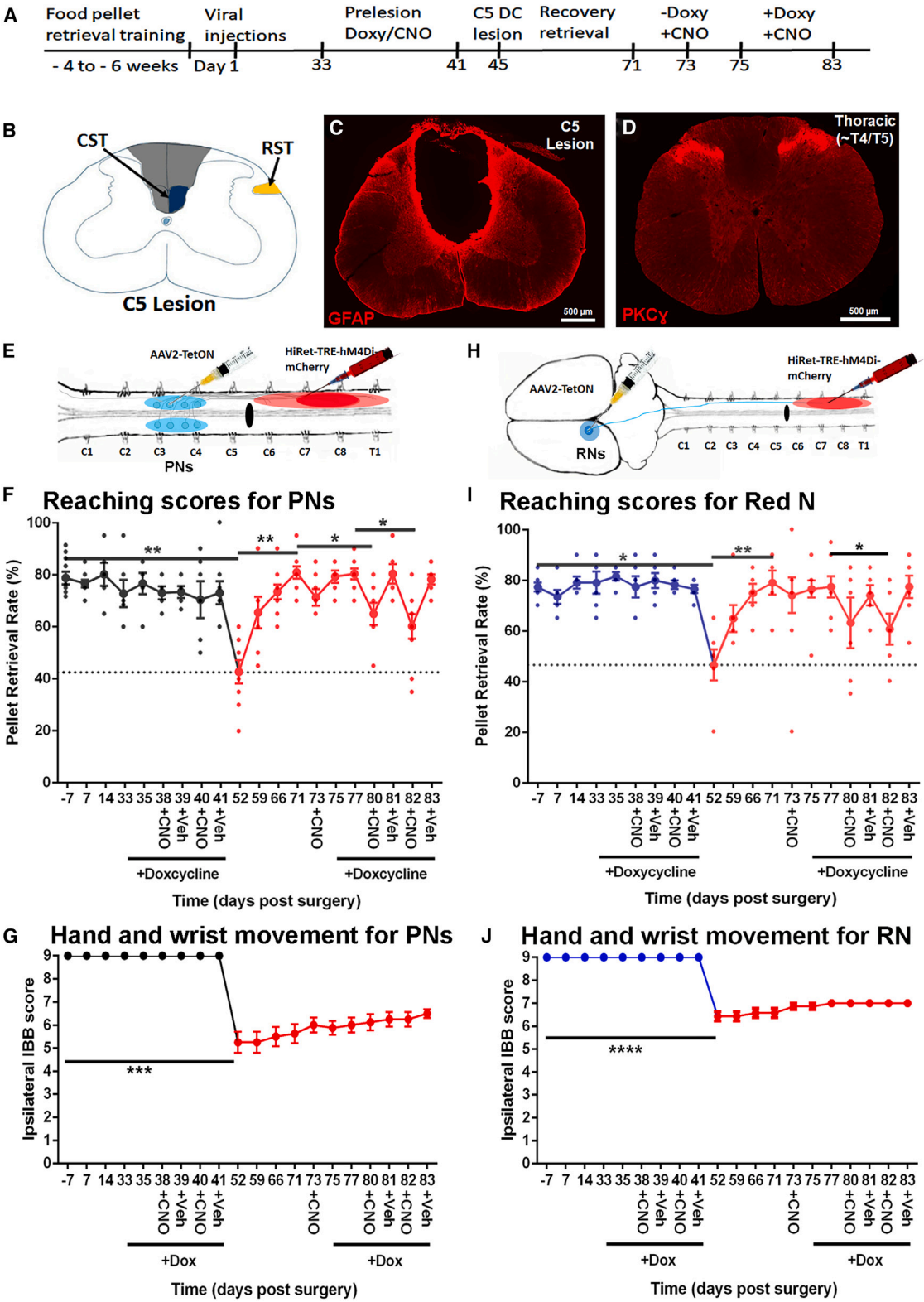

Figure 3. Recovery of reaching is mediated by C3/C4 propriospinal and rubrospinal neurons after C5 dorsal funiculus lesions

(A) Experimental timeline describing training, viral injections, lesion time point, recovery, CNO pre-induction of hM4Di and post induction for silencing of individual populations.

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C3-C4 PNs alone (Figure 3F) showed an 18% and 24% decrease in reaching behaviors after administration of CNO ($F(19,108) = 4.508$, $p < 0.0001$; Sidak's post hoc for day 71 vs. 80, $p = 0.0117$ and day 77 vs. 82, $p = 0.0021$; Figure 3F). In these rats, reaching attempts resulted in more pronounced mistargeting of the pellet. Rats would often reach with digits extended, but over- or under-reach the pellet often. In multiple instances the over-reach sent the pellet flying off the shelf. When they did target the pellet, they would pronate and grasp the pellet, but rarely lift it, instead dragging it off the shelf.

Likewise rats expressing hM4Di targeted to the RSNs (Figure 3I) showed a 19.3 and 20.7% decrease in reaching behaviors with the second administration of CNO being statistically significant ($F(19,100) = 2.55$, $p = 0.0014$; Sidak's post hoc for day 71 vs. 80 $p = 0.062$ and day 77 vs. 82, $p = 0.0366$; Figure 3I). Silencing of the red nucleus led to a slightly different response, in which the rats targeted the pellet well, but often failed to pronate when attempting to grab the pellet. In many instances they prematurely extended their digits before reaching the pellet, brushing it off the shelf usually outside the chamber. When they did hook or grasp the pellet, again, they mostly dragged it off the shelf. These data indicate that both C3/C4 propriospinal and rubrospinal pathways contribute to recovery of reaching behavior after cervical injury. In addition, since dorsal funiculus lesions did not result in 100% loss of targeted reaching and grasping, other axonal tracts partially or completely unaffected by this injury (e.g., reticulospinal, lateral or ventral CST) also contribute to reaching and grasping behavior in the rat.

In primates, silencing of C3/C4 propriospinal neurons reduces finger grasping and dexterity, however in mice, hand and finger movement are influenced by dI3 neurons and thought to function independently of C3/C4 propriospinal neurons.[37] To examine if C5 lesions results in deficits in hand and wrist movements, followed by recovery with training, we used the Irvine, Beatties and Bresnahan (IBB)[32,33] forelimb recovery scale. Lesions at C5 resulted in a significant reduction in digit, hand and wrist movements on the ipsilesional side with no statistically significant recovery (Figure 3G and 3J). Doxycycline expression and subsequent CNO activation of hM4Di in either the C3/C4 PNs or RNs had no effect on digit, hand or wrist patterns while manipulating cereal. These results show that unlike monkey, C3/C4 propriospinal neurons do not contribute to finger dexterity or grasping in rats.

Rehabilitation induced forelimb recovery is mediated by both C3/C4 propriospinal and rubrospinal neurons

Silencing either C3-C4 PN or RNs accounted for only about 50% of the recovery occurring after lesioning and training. Since RNs have previously been shown to synapse on C3/C4 PNs in addition to motor neurons in the spinal cord, we examined if these pathways functioned independently (in parallel) or together in series. If the pathways function in parallel than silencing both should have an additive effect and reduce the reaching success further toward post-lesion lows; however, if in series silencing both pathways would result in a reduction of function no greater than either alone. For this study, all rats followed the experimental timeline for injections, lesioning, training and silencing of both C3/C4 PNs and RNs (Figures 4A and 4B). Silencing of both pathways lead to a significant decrease in reaching ($F(7,84) = 3.302$, $p = 0.0038$). Similar to previous results, C5 lesioned rats showed deficits in their ability to successfully retrieve food pellets (Sidak's post hoc for day 35 vs. 66, $p = 0.0049$) that significantly recovered to baseline levels 28 days post-injury (Sidak's post hoc for day 66 vs. 87; $p = 0.0092$; Figure 4C) with continued training. Administration of doxycycline started after control CNO injections on day 89 and continued for eight days. Five and seven days after induction of hM4Di expression targeted to both C3-C4 PNs and RNs, the administration of CNO induced deficits in reaching behavior of 32 and 37% below recovery levels (Sidak's post hoc for day 91 vs. 94, $p = 0.0032$ and day 95 vs. 96, $p = 0.0122$; Figure 4C). These rats showed good forelimb targeting prior to lesioning (day 35; Figure 4D) and after completion of rehabilitative training (day 87; Figure 4F), but repeatedly mistargeted the pellet 7 days post-injury (day 66; Figure 4E) or when treated with doxycycline and CNO (day 94; Figure 4G), leading to multiple reach attempts and misses or immediately knocking the pellet off the shelf. Their reaching deficits were very similar to those observed after lesioning, however, with fewer incidents of missing the slit and hitting the shelf during the initial reaching attempt. The majority of errors were associated with miss targeting or sideswiping the pellet during the reach. As observed with silencing of individual pathways, silencing both C3/C4 PNs and RNs did not affect hand and wrist movements, which only marginally recovered after C5 lesions.

To determine if silencing both pathways had a significant additive effect, we first calculated the individual differences in

(B) Illustration showing lesion area shaded in gray.
(C) Representative section showing C5 dorsal funiculus lesion stained by GFAP.
(D) Representative section showing loss of PKC-gamma staining of CST in T4 spinal cord section.
(E) Illustration showing injection procedure for expressing hM4Di in propriospinal neurons.
((F) Graph showing pellet retrieval scores prior to lesioning (black) and after C5 DC lesions (red). Significant deficits in pellet retrieval were observed 7 days after DC lesions ($p > 0.001$ any black time point compared to day 52 of trial) with significant recovery back to baseline by day 71 of trial (comparison of day 52 to day 71, $p = 0.0003$). After induction of hM4Di by administration of doxycycline, CNO induced a decrease of 18% at day 80 ($p = 0.0117$) and 24.1% at day 82 ($p = 0.0021$) when compared to vehicle administration.
(G) After C5 DC lesions all rats showed persistent deficits in hand, wrist, and digit movements (−7 vs. 52 days; $p = 0.0001$), which only marginally recovered (52 vs. 71 days; $p = 0.3119$) and showed no response to CNO treatment.
(H) Illustration showing injection procedure for expressing hM4Di within neurons of the red nucleus.
(I) Graph showing pellet retrieval scores prior to lesioning (blue) and after lesioning (red) with CNO inducing a 19.3% ($p = 0.062$) and 20.6% ($p = 0.0366$) reduction at days 80 and 82, respectively, when compared to vehicle injections.
(J) As with panel G, deficits in hand, wrist and digit movements did not resolve over time or change with CNO treatment. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ Data is mean ± SEM, C3/C4 PNs $n = 8$, RNs $n = 6$. Scale bars in panels C and D are 500 μm. Also see Figure S2.

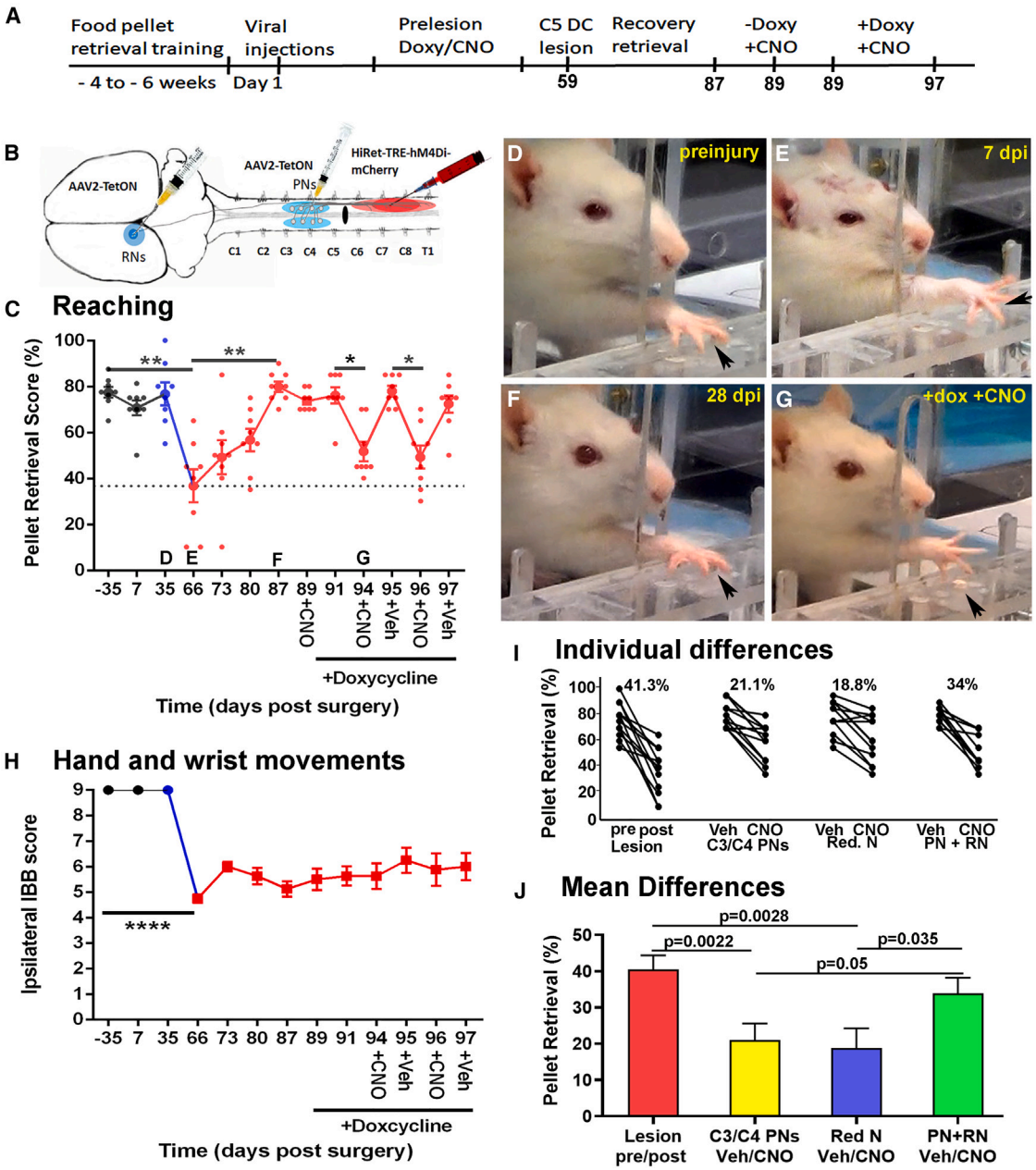

Figure 4. Silencing both C3/C4 PNs and RNs together have a combined reduction in the recovery of forelimb reaching

(A) Experimental timeline describing training, viral injections, lesion time point, recovery, CNO pre-induction of hM4Di and post induction for simultaneous silencing of both C3/C4 PNs and RNs populations.

(B) Illustration showing injections procedure for expressing hM4Di in both propriospinal and rubrospinal neurons and lesion site (black).

(C) Graph showing pellet retrieval scores prior to lesioning (blue) and after C5 DC lesions (red). Significant deficits in pellet retrieval were observed 7 days (day 66, E) after DC lesions ($p > 0.001$ any black time point compared to day 52 of trial) with significant recovery back to baseline by day 71 of trial (comparison of day 52 to day 71, $p = 0.001$). After doxycycline induction of hM4Di, administration of CNO induced a 32% ($p = 0.0127$) and 37% ($p = 0.043$) reduction in pellet retrieval at 94 and 96 days, when compared to vehicle control injections. Repeated measures one-way ANOVA was significant: F (2.739, 19.17) = 12.59, $p = 0.0001$. Sidak's post hoc analysis done for multiple comparisons.

(D–G) Individual video frames of representative reaching attempts at pellets (arrowheads) at timepoints indicated by letters in panel C. The entire video is in supplemental video.

(H) Deficits in hand, wrist and digit movements did not resolve over time or change with CNO treatment.

(I) Differences in single pellet retrieval between pre- and post-lesion or vehicle and CNO treatment for each group.

(J) T-test comparison between different treatment groups. $^{**}p < 0.01$, $^{*}p < 0.05$, $^{***}p < 0.001$, Data is mean ± SEM, $n = 8$.Also see Video S1 and Figure S2.

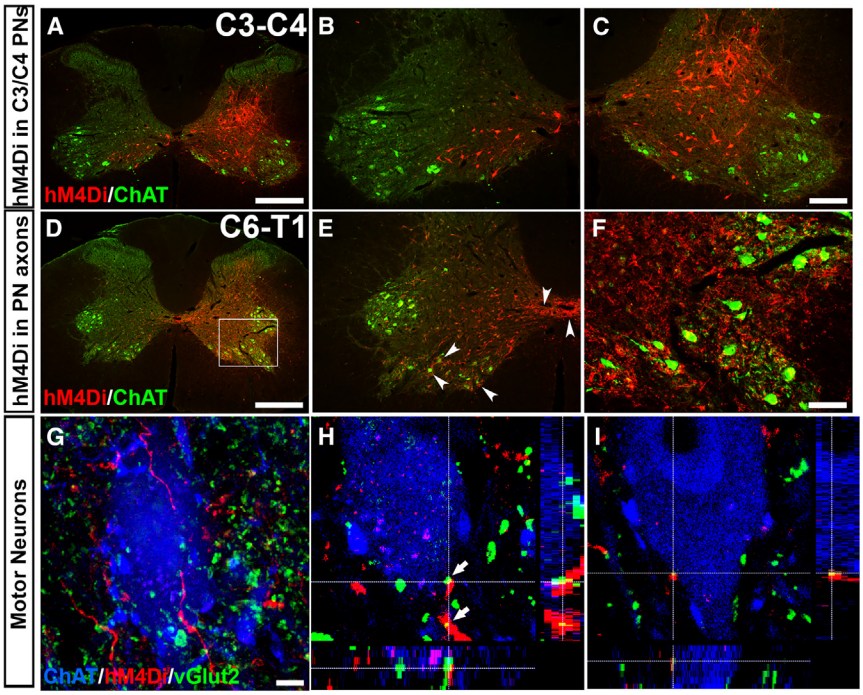

Figure 5. hM4Di+ specifically labels C3-C4 PNs and axon terminals onto motor neurons in the C6-T1 spinal cord

(A) Spinal cord C3-C4 sections showing bilateral distribution of hM4Di-mCherry expression in the intermediate gray matter and ChAT+ MNs.

(B) Higher magnification of contralateral C3-C4 spinal cord.

(C) Higher magnification of ipsilateral C3-C4 spinal cord. No overlapping expression of hM4Di and ChAT was observed in C3-C4.

(D) C8 spinal cord section showing hM4Di+ C3-C4 PN axon terminals within the intermediate zone and ventral horn.

(E) Higher magnification of contralateral C8 spinal cord showing hM4Di axons crossing the midline toward ChAT+ MNs.

(F) Higher magnification of ipsilateral C8 spinal cord showing numerous hM4Di+ axons within gray matter surrounding ChAT+ MNs.

(G) Maximum intensity projection (MIP: 20 μm) of confocal z stack showing hM4Di+ C3-C4 axon terminals co-expressing vGlut2 near ChAT+ MNs.

(H and I) Orthogonal sectioning showing hM4Di+/vGlut2+ C3-C4 PN axon terminals contacting ChAT+ C8 MN somas. Scale bars in (A) and (D) is 500 μm; (B), (C), and (E) are 200 μm; (F) is 100 um (G) is 10 μm.

reaching success between vehicle and CNO treatment for C3/C4 PNs, RNs, and both pathways as wells between prelesion and postlesion results (Figure 4I). Silencing C3/C4 PNs or RNs individually lead to a 21.1% and 18.8% respective decrease in the success of reaching and retrieval of food pellets that was not statistically different between these two groups ($t(30) = 0.3159$, $p = 0.754$. Whereas, silencing both pathways simultaneously resulted in a 34% reduction, which was not statistically different ($t(28) = 1.16$ $p = 0.26$) from the decrease of 41.3% caused by the lesion, demonstrating that pellet reaching success scores after silencing both C3/C4 PNs and RNs together were similar to the lesion. However, silencing either the C3/C4 PNs ($t(34) = 2.045$ $p = 0.0487$) or RNs ($t(26) = 2.223$ $p = 0.035$) individually were statistically different from silencing both C3/C4PNs and RNs together, showing that these pathways act in parallel and not in series to activate motor neurons (Figure 4J) after lesioning the CST and rehabilitative training.

Localization of C3/C4 propriospinal axonal terminal onto motor neurons in the C6–T1 spinal cord and lateral reticular neurons

To verify hM4Di expression is localized to the appropriate neurons, cervical spinal cord, midbrain and brainstem tissue was dissected and sectioned. As expected, hM4Di expression localized to the RNs and PNs within the C3-C4 spinal cord (Figures 2 and 5) with similar distribution and labeling as previously described.[12,22,31] Since, expression of hM4Di within spinal motor neurons would negatively affect reaching and grasping, we examined expression within ChAT positive cells. Spinal motor neurons in either the C3/C4 (Figures 5A–5C) or the C6-T1 (Figures 5D–5F) or any motor neurons identified within the spinal cord failed to show hM4Di expression. Although, we did not identify any hM4Di+ spinal motor neurons, we did observe numerous axon terminals from hM4Di-mCherry positive C3-C4 PN within the C6-T1 region ipsilateral to the preferred forepaw (Figure 5). Interestingly, some axon terminals also crossed the midline, entered the contralateral spinal cord and terminated near C6-T1 forelimb MNs (Figure 5E). PN axon terminals are primarily glutamatergic and express the excitatory presynaptic marker vGlut2.[21,24] Confocal images of hM4Di$^+$/vGlut2$^+$ PN axon terminals were identified at the somas of ChAT$^+$ C6-T1 MNs (Figure 5G and 5I) and confirmed using orthogonal sectioning from individual motor neurons (Figures 5H and 5I). These data show that hM4Di-mCherry+ C3/C4 axons directly synapse onto spinal motor neurons, reinforcing motor drive in the absence of CST connections.

Propriospinal neurons expressing hM4Di localized to Rexed laminae IV–VIII in the intermediate gray matter similar to other studies in cat[38,39] and monkey,[28,40] and for V2a PNs in mice.[24] Previous studies identified V2a, dl3 and V3 glutamatergic neurons within the cervical PNs to have a bifurcated axon with one branch terminating on C6-T1 spinal motor neurons and the other extending into the LRN, which in turn sends axons into the cerebellum.[41] To verify that some of the labeled C3/C4 PNs were these interneurons, we examined their synaptic connectivity within the LRN. We observed hM4Di-mCherry$^+$ expression in C3-C4 PN axon terminals in both the C6-T1 ventral horn and the LRN. Numerous hM4Di$^+$ axon terminals specifically targeted the caudal brainstem at the location of the LRN (Figures 6A and 6B). Confocal imaging showed numerous hM4Di$^+$/vGlut2$^+$ axon terminals around LRN neurons with synaptic contacts on somas (Figures 6C–6F). These data indicate that the hM4Di-mCherry+ neurons labeled within the C3/C4 spinal cord show the characteristic features of V2a,

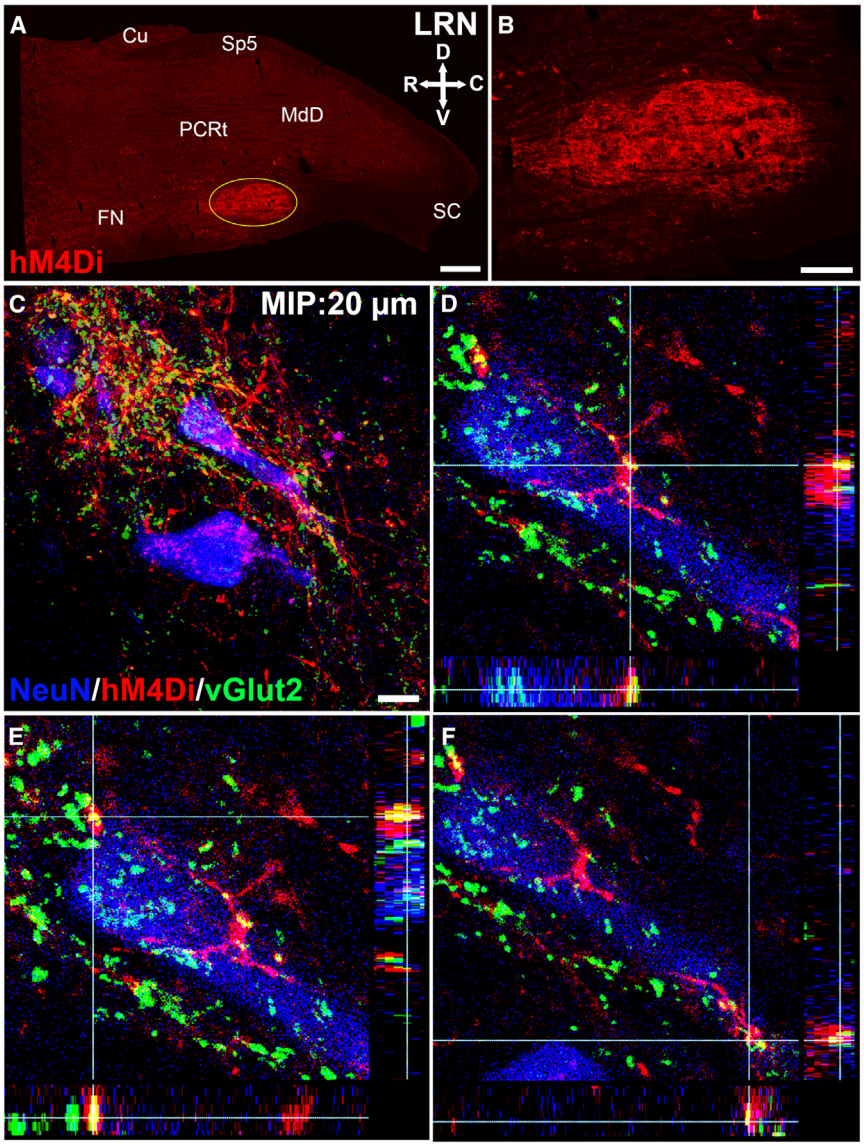

Figure 6. hM4Di expressing C3-C4 PN axon terminals in LRN

(A) Brainstem section stained for mCherry showing hM4Di expressing axon terminals from C3/C4 propriospinal neurons almost exclusively targeting the LRN (red labeling surrounded by yellow oval), similar area of illustration in Paxinos and Watson 7th edition, figure 171.

(B) Higher magnification image of LRN showing hM4Di+ C3-C4 PN axon terminals.

(C) Maximum intensity projection (MIP: 20 μm) confocal image showing hM4Di+/vGlut2+ PN axon terminals around NeuN+ LRN neurons. Orthogonal sectioning of z stack images showing hM4Di+/vGlut2+ PN terminals on NeuN+ LRN neuron (D), (E) somas and (F) dendrite. Red = hM4Di-mCherry, Green = vGlut2, Blue = NeuN. Scale bar in (A) 500 μm, (B) 200 μm and (C) 10 μm. Cu: Cuneate Nucleus; Sp5: spinal trigeminal tract; SC: spinal cord; MdD: Medullary Reticular nucleus, dorsalis; PCRt: Parvicellular reticular nucleus; FN: Facial Nucleus.

dI3 and V3 interneurons thought to provide an internal motor copy to adjust movements in real time.[24]

Severed CST fibers sprout into red nucleus and C3-C4 spinal cord

Sprouting of axonal fibers is known to occur post-injury within the brain and spinal cord. Severed CST axons sprout post-injury onto propriospinal interneurons (PNs) to mediate rehabilitation-directed recovery of locomotion.[16,29,42] After pyramidotomy sprouting of CST fibers into subcortical nuclei permit recovery of forelimb function.[12,36] Sprouting of CST axons onto these spared nuclei or neuronal populations are thought to induce relays around the lesion to support functional recovery. We examined if severed CST axons can undergo sprouting into the C3-C4 spinal cord and red nucleus with dorsal funiculus lesions. To examine sprouting, we injected BDA into the sensorimotor forelimb cortex contralateral to the preferred forepaw in all animals. Sprouting was examined in three cohorts of rats: (1) normal animals with no pellet retrieval training and no dorsal funiculus injury, (2) C5 dorsal funiculus lesions with no training, and (3) C5 dorsal funiculus lesions with rehabilitative training (single pellet retrieval and IBB) post-injury and expression of hM4Di within either the spinal cord or red nucleus. Lesioned rats that did not receive rehabilitative training showed decreased BDA-labeled CST fiber sprouts within the gray matter relative to uninjured controls ($p < 0.0001$) (Figures 7A, 7B, and 7D). Rehabilitative reach training after C5 lesions increased CST fiber sprouting into the region of C3/C4 PNs when compared to lesion with no training ($p = 0.0116$) (Figures 7B and 7D). In the red nucleus, lesioned animals with no training showed significant decreases in labeled CST fiber sprouts when compared to normal rats ($p = 0.0079$) (Figures 7E, 7F, and 7H). Rats with the C5 dorsal funiculus lesion and training showed a similar density of CST fiber within the red nucleus when compared to normal controls, but over 2-fold higher than lesioned rats with no training ($p = 0.0058$) (Figures 7F and 7H). These data further support the importance of rehabilitative training to enhance recovery and increase anatomical plasticity of severed cortical fibers to promote functional recovery. In addition, the data show that CST axons after C5 dorsal funiculus lesion sprout rostral to the injury with training and promote connections onto secondary pathways bypassing an injury to promote skilled forelimb reaching and grasping of food pellets. Interestingly, these secondary pathways, on their own, do not show a significant contribution to reaching and grasping in normal rats.

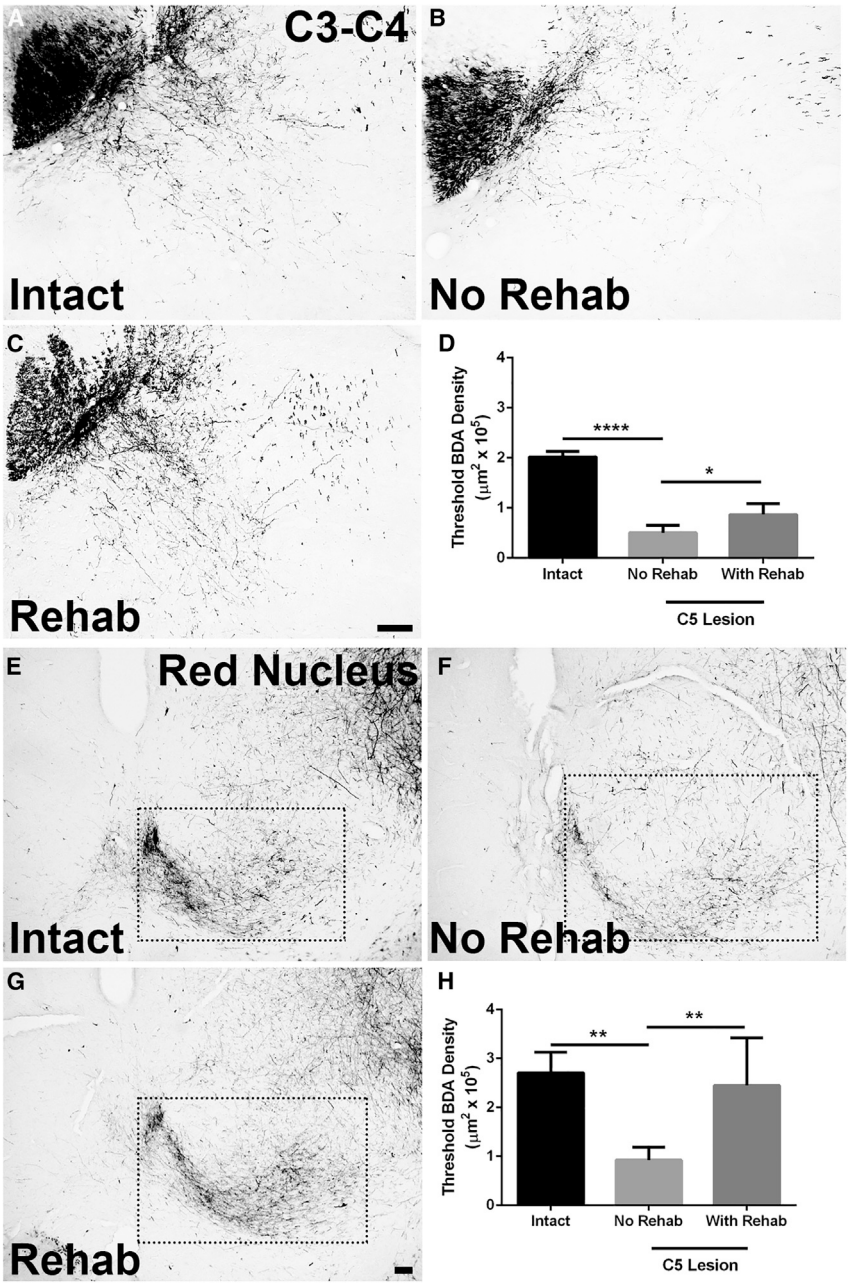

Figure 7. CST fiber sprouting in C3-C4 spinal cord and red nucleus post-injury is dependent on rehabilitative training

(A) Corticospinal axons extend into the region of the C3-C4 propriospinal neurons.

(B and C) Six weeks following C5 dorsal funiculus lesion, animals that underwent rehabilitative behavioral training showed increased sprouting of corticospinal axons into the C3-C4 spinal cord (C), whereas those that did not undergo training showed no sprouting (B).

(D) Quantification of thresholded BDA fiber density within the C3-C4 gray matter was statistically significant by one-way ANOVA; $F(2, 12) = 75.69$; $p < 0.0001$. Sidak's post hoc multiple comparison was significant: intact vs. lesion no rehab, $p < 0.0001$; and lesion with no rehab vs. lesion with rehab; $p = 0.0116$.

(E–G) Corticospinal axons also extend into the red nucleus in intact, non-injured rats. Six weeks following C5 dorsal funiculus lesions with rehabilitative training show extensive sprouting into the red nucleus (G) when compared to a similar group not receiving rehabilitative training (F).

(H) Quantification of thresholded BDA fiber density in the red nucleus was statistically significant by one-way ANOVA; $F(2, 12) = 10.62$; $p = 0.0022$. Sidak's posthoc multiple comparison was significant: intact vs. lesion no rehab, $p = 0.0079$; lesion with no rehab vs. lesion with rehab; $p = 0.0058$. Data is mean ± SD; $n = 3–6$. $^{**}p < 0.01$, $^{*}p < 0.05$. Scale bars in (A –C & E–G) 100 μm.

DISCUSSION

Many studies show the importance of propriospinal neurons in establishing relays that bypass the lesion to contribute to the recovery of locomotion. Severed supraspinal axons are thought to directly synapse or sprout onto PNs to relay motor commands past the lesion to caudal networks ultimately increasing motor neuron activation.[16–18,20,43] Previous, studies have examined the ability of PNs to contribute to recovery of locomotion, but not skilled forelimb patterning. Our results demonstrate the important contribution of both the RN and C3-C4 PNs on recovery of target-directed reaching behavior in a rodent model of cervical SCI. In addition, the data illustrates a greater loss in motor recovery when several major pathways are simultaneously silenced, indicating they provide different contributions to motor patterning to mediate successful retrieval of food pellets.

Our first goal was to determine if neuronal silencing of C3-C4 PNs, RSNs or both affect forelimb reaching and grasping behaviors in rodents. We chose these two specific populations based on retrograde tracing studies done with HiRet-GFP[31] and information in the literature indicating their involvement in recovery of forelimb function.[6,23] Neuronal silencing of C3-C4 PNs and RSNs either individually or in combination using inhibitory DREADDs in uninjured animals did not produce any forelimb functional deficits in any of our behavioral paradigms. Even though neuronal silencing of C3-C4 PNs in uninjured primates using tetanus toxin resulted in reduced finger dexterity, such impairments occur only over a short time period until direct cortico-motoneuronal pathways could compensate.[28] Even simultaneous silencing of both C3-C4 and RSN pathways failed to produce any observable deficit in single pellet retrieval, indicating these pathways play a minor role in targeted reaching and grasping and act in a supportive role to adjust movement errors relayed through the corticospinal tract (CST).

The pre-injury results are consistent with the idea that many motor pathways may participate in secondary adjustment to movement and thus provide compensatory redundancy if one component is damaged. The cortex is the major driver for many voluntary motor movements. After lesioning, subcortical nuclei[2,4,44] along with other tracts can compensate for loss of function mediated by the CST.[9,11,15,45–47] Further, neuronal silencing or even injury to individual spinal tracts may not produce significant deficits without sensitive measures. For example, examination of behavioral performance after lesions to the rubrospinal tract or red nucleus induced transient deficits in arpeggio movements during protonation in the single-pellet reaching test.[48,49] However, such deficits are relatively minor especially since the CST directs fine motor control of the forelimb during reaching and grasping. However, combined lesions to both the CST and RST result in significant errors during voluntary movements.[13,50] These results indicate supportive pathways can undergo adaptive compensation to create functional alternative motor pathways after spinal cord lesions.

Many severe cervical SCI models affect multiple descending motor tracts (e.g., dorsal hemisection, unilateral hemisection, contusion). Loss of several major tracts innervating the rodent forelimb motor pools induce severe deficits in gross forelimb function. Additionally, dorsal lesions interrupt conscious tactile and proprioceptive information. With severe injuries that lesion multiple forelimb motor and sensory pathways, recovery of target-directed reaching behavior typically does not occur since most of the ipsilateral pathways are lost.[35] However, in C5 dorsal funiculus injury models, many animals recover close to baseline measurement levels by four to six weeks post-injury, with training.[8,15,51] This injury spares the dorsolateral funiculus of the spinal cord containing the RST and the lateral CST.[52–54] In addition, these lesions spare the ventrolateral funiculus containing extrapyramidal fibers (ex. Ipsilateral CST, reticulospinal, vestibulospinal, tectospinal) and C3-C4 PN fibers found near the ventral grey-white matter interface.[31] In the absence of dorsal CST and conscious proprioceptive information, these pathways provide some support for forelimb use, since we never observed complete loss of reaching and about a 60% success in single pellet retrieval remains. Interestingly, the recovery of forelimb function gradually increased over the training period of 4 weeks indicating that this recovery required adaptation of existing pathways bypassing the lesion.

Once our animals recovered reaching behavior, we induced the expression of inhibitory DREADDs and activated them with CNO. We observed deficits in target-directed reaching behavior in animals after silencing of RSNs. Previous publications demonstrated RSN to promote recovery of forelimb reaching after pyramidal tract lesions; however, their lesion deficits were much worse and recovery not as robust as with C5 DC lesions.[12] Pyramidotomies not only eliminate CST innervation to the spinal cord, but also most of the medulla, which contains the LRN, gigantocellular reticular nucleus and ventral medial reticular formation, all thought to be involved in skill forelimb movements.[18,23,55] Silencing of the C3-C4 PNs also resulted in statistically significant mistargeted reaching attempts, with a decrease slightly more than observed by silencing RNs alone. Silencing both pathways showed a more robust deficit, nearly twice as large as each individually pathway and similar to the loss mediated by the lesion itself. This data indicates that recovery of motor function after SCI requires adaptation of several different pathways, functioning in concert, to produce sufficient drive and coordination for proper forelimb targeting.

C3-C4 PNs not only relay motor commands, they also receive monosynaptic inputs from Ia sensory afferents from the forelimb.[25,56] Conscious proprioception travels through the dorsal columns and ascends to the somatosensory cortex and supraspinal structures for identity of limb position. In the cat, loss of feedback inhibition after lesioning dorsal column sensory afferents prevents termination of forelimb muscles causing hypermetria during reaching movements.[25,56] In our rats, we observed mistargeting upon forelimb reaching toward a pellet leading to immediate knockoffs or an inability to appropriately correct a single reach movement during DREADD activation. This behavioral phenotype was similar to what we observed the first week post-injury. It is possible that during rehabilitative training compensatory mechanisms integrate both sensory and motor information locally[57] or through the cerebellum. The LRN is composed exclusively of projection neurons to the cerebellum, forming a loop extending back to the brainstem.[41] The loss of ascending dorsal column sensory information would restrict sensory motor integration to either the cerebellum or local interneurons. Once silenced, convergence of supraspinal motor information through C3-C4 PNs is temporally interrupted failing to update supraspinal centers regarding limb position and trajectory, thus resulting in dysmetria.

An extensive body of work by Alstermark and Isa illustrates the importance of C3-C4 PNs on goal-directed reaching and grasping behaviors in both cats and primates. Using electrophysiological stimulation of the CST after C5 lesions to the dorsolateral funiculus, Alstermark et al. demonstrated re-routing of CST and RST inputs to forelimb MNs through C3-C4 PNs.[23] In addition, Isa's group used tetanus toxin silencing of C3-C4 PNs to demonstrate this indirect corticomotoneuronal pathway affects finger dexterity in uninjured and injured primates.[28,58] In our studies, we observed deficits in target-directed reaching only after C5 dorsal funiculus injury, but not uninjured rodents. These results are likely due to species differences (primate vs. rodents and cats) in cortical pathway transmission for forelimb innervation. These studies highlight the importance of brainstem and spinal pathways as bypass circuits to allow supraspinal transmission to denervated MNs.

Limitations of the study

This study is limited to lesions affecting the CST in the cervical spinal cord, since a dorsal funiculus lesion was used in the study. Other forms of spinal injury such as moderate or severe contusion would injure more or different pathways and circuits, possibly the ones contributing to recovery in this model. CST lesions primarily cause deficits in skilled forelimb reaching and grasping and digit dexterity, but not locomotion. Since the severity and location of the spinal cord location can dramatically affect the number and types of pathways damaged, further studies are required to examine circuit modifications leading to recovery in other spinal cord injury models.

RESOURCE AVAILABILITY

Lead contact

Further information concerning reagents or methods and all requests for plasmids and viruses should be directed to and will be fulfilled by the lead contact Dr. George M. Smith (george.smith@temple.edu).

Material availability

pBOB-tre-hM4Di-mCherry plasmid is available upon request from Dr George Smith.

Data and code availability

- The datasets from this study are available from the corresponding author upon request.
- No novel code was generated in this study.
- Any additional information required to reanalzse the data reported in this paper is available from the lead contact upon request.

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AUTHOR CONTRIBUTIONS

I.S.S., K.M.K., N.A.S., I.P.J., C.L., and J.C. conducted the experiments or analyzed the behavioral data. I.S.S., X-M.X., L.G.K., and G.M.S. designed the experiments and wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-DsRed	Clontech	Cat# 632496; RRID: AB_10013483
Mouse anti-NeuN	Millipore	Cat#MAB377; RRID: AB_2298772
Goat anti-ChAT	Millipore	Cat#AB144P; RRID: AB_2079751
Guinea Pig anti-vGlut2	Millipore	Cat#AB2251; RRID: AB_1587626
Rabbit anti-PKC-γ	Abcam	Cat# 71558 and AB181558; RRID: AB_3662843
Mouse anti-GFAP	Sigma	Cat# G3893; RRID: AB_477010
Bacterial and virus strains		
HiRet-CMV-GFP	Shriners Neural Repair Viral Core	N/A
pAAV2-CMVrTAV16	Shriners Neural Repair Viral Core	N/A
HiRet-tre-hM4Di-mCherry	Shriners Neural Repair Viral Core	N/A
Chemicals, peptides, and recombinant proteins		
Clozapine-N-Oxide	Tocris biotech	Cat #4936
Doxycycline	Sigma	Cat # D9891
Biotinylated dextran amine (10,000 MW)	Molecular probes (ThermoFisher)	Cat # D1956
Experimental models: Organisms/strains		
Rat (Sprague Dawley)	Charles River laboratories	
Recombinant DNA		
p FuG-B (HiRet) lentivirus Rabies-G/VSV chimera	Kato et al., 2011	
pAAV2-CMVrTAV16	This paper	
pBOB-tre-hM4Di-mCherry	This paper	hM4Di-mcherry (Addgene #44362)
Software and algorithms		
Nikon Elements 3.0	Nikon	https://www.microscope.healthcare.nikon.com/products/software/nis-elements
GraphPad Prism 7	Graphpad	https://www.graphpad.com/scientific-software/prism/
Stereoinvestigator software	MBF Bioscience	https://www.mbfbioscience.com/products/stereo-investigator
Zeiss Axiovision software	Zeiss	https://www.micro-shop.zeiss.com/en/us/softwarefinder/
Leica LASX software	Leica	https://www.leica-microsystems.com/products/microscope-software/p/leica-las-x-ls/

EXPERIMENTAL MODEL AND STUDY PARTICIPANT DETAILS

Animals

All surgical and animal care protocols were approved by the Temple University School of Medicine's Institutional Animal Care and Use Committee and performed per the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Female and male Sprague-Dawley rats (65-75 d, 200-224 g; Harlan Laboratories) were housed two per cage, on a 12-hour light-dark cycle with food and water provided *ad libitum*. Animals were allowed 7 days of acclimatization prior to any experimental procedure.

METHOD DETAILS

Viral vectors

Synaptic silencing of specific neuronal pathways was achieved with a two-viral vector system utilizing tetracycline-inducible expression of the inhibitory DREADD (hM4Di).[59] HiRet-tre-hM4Di-mCherry plasmid was constructed using the lentivirus backbone plasmid

(pCSC-SP-PW-GFP, Addgene plasmid # 12337, a gift from Inder Verma;).[60] To generate this plasmid, the original CMV promotor and GFP cassette was excised and replaced with a tetracycline responsive element (TRE) containing inhibitory DREADD (hM4Di) fused with mCherry using the ClaI/PmeI sites within the original plasmid. To verify cDNA integrity and protein expression, the plasmid was sequenced and examined for mCherry fluorescent activity and size determination by Western blot after transfection into HEK 293 cells.

To generate HiRet lentivirus, the HiRet-tre-hM4Di-mCherry plasmid was packaged using the HiRet rabies virus fusion envelope glycoprotein G (FuG-B; provided by Dr. Kobayashi) and plasmids pMbl and pRev. Rabies virus glycoproteins allow lentivirus uptake at the synapse and retrograde transport with high efficiency into neuronal somas.[28,61] Plasmids were transfected into HEK293T cells using the $CaPO_4$ method and allowed to incubate overnight. The supernatants were collected 72 hours after transfection and concentrated by ultracentrifugation and the viral pellet was aliquoted and stored at −70°C until further use. Purified virus was suspended in Tris buffer containing rat albumin and mannitol. Lentiviral titers were determined using a P24 HIV-1 ELISA kit. All lentiviruses were at least 2 x 10^7 TU/μL infectivity. A highly efficient retrograde gene transfer (HiRet) lentiviral vector (provided by Dr. Kobayashi)[30] was used in conjunction with an adeno-associated vector (AAV-TetOn) containing a tetracycline transactivator and subcutaneous injection of doxycycline antibiotic.

The plasmid pAAV-TetOn was transfected into HEK293T cells with the addition of pHelper containing adenoviral functions and pRC containing *rep* and *cap* genes. 293T cells were grown to 70-80% confluence at which point they were transfected with packaging plasmids using PEI (Polyethylenimine, linear, MW-25k, Warrington, PA). Three days after transfection, the cell lysates and the supernatant were harvested and 40% PEG 8000 was added to precipitate crude virus for 2 hours. Then the viruses were purified by double-centrifugation with cesium chloride (CsCl) and the isolated virus was dialyzed in 0.1M PBS/5% sorbital overnight.[62,63] The titers of AAV-TetOn used in this study were determined by infecting fibroblast cells. The use of fibroblast cells allows for AAV2 vector to induce rapid transgene expression to examine expression of transgenes. The fraction with the highest expression was used in the following *in vivo* studies. AAV2-TetOn titers used in this study were 1.2 x 10^{13} GC/mL as determined by quantitative real-time PCR. Lentivirus and AAV2 were aliquoted and stored in -80°C.

Drug administration

To induce expression of hM4Di, doxycycline (30 mg/kg, s.c.) was administered daily for behavioral assessments to determine effects of synaptic silencing. Clozapine-n-oxide (CNO; 4 mg/kg, i.p.) was administered 20-30 minutes prior to behavioral assessments to determine effect of inhibitory DREADD activation on forelimb function including reaching and grasping behaviors. Vehicle injections (0.5% dimethyl sulfoxide in saline, i.p.) were given the following day to examine if behavioral recovery return to baseline performance. Doxycycline was also administered prior to euthanasia to visualize neuronal mCherry expression upon histological analysis.

Behavioral assessments

Forelimb motor function was assessed with the following paradigms: limb asymmetry (cylinder test), the Irvine, Beattie, Bresnahan (IBB) forelimb test and the single pellet reaching task. Baseline measurements were taken on all animals prior to surgical procedures. Experimenters blinded to treatment completed all assessments and analysis.

The single-pellet reaching task measures reaching and grasping behaviors using the hand, wrist and digits.[14,64] Animals are placed into a plexiglass box with a shelf where chocolate flavored sucrose pellets are located (Dustless Precision Pellets, chocolate flavor, sucrose #F0025, Bioserv, Flemington, NJ). Animals that learn the task of reaching-to-grasp-to-retrieve and eat food pellets with a preferred forelimb are subsequently trained to go to the back of the box to initiate a new presentation of a pellet. All rats were required to move to the back of the cage prior to the placement of the next pellet during testing. Fully trained animals have baseline measurements averaged over three consecutive trial days. For testing, rats were given 10 pellets for prescoring and 20 for scoring. Rats that fail to achieve an average reaching success of 60% or switch paw handedness are removed from the study. The successful pellet retrieval rate percentage was determined by counting the total number of pellets obtained and eaten out of 20 test pellets. We also counted the number of attempted reaches prior to either gasping or knocking the pellet off the shelf. The total number of reaching attempts for the 20 pellets, whether successful or not, were also recorded.

Videos of 2 reaches for each rat were examined and rated for qualitative features of the movement.[13] All measures were done in 0.25% speed to examine each movement pattern. The movements were rated according to the following scale: (1) The limb is lifted off the floor and swung across the body. (2) The hand is in a flexed position with palm facing the body as the limb is lifted. (3) during the reaching movement the elbow is adducted, and forearm aligned so the paw located just under the mouth. (4) The limb is advanced through the opening in the box and advanced toward the food pellet. (5) The hand opens and digits extend outward to grasp the pellet. (6) Just prior to grasping the elbow is abducted and paw pronates in preparation to grasp the pellet. (7) The food is grasped by closure of the digits. (8) The paw is supinated by 90° and elbow adducted as the pellet is lifted and moves towards the mouth. (9) Just prior to eating, the rat sits, and the paw is supinated further as the food moves towards the mouth. (10) the hand opens and releases the pellet into the mouth. Each of the 10 categories is also given a rating scale to be judged as normal, slightly impaired, or severely impaired; though in some cases animals given the same score could have different levels of severity within a particular category.

The IBB measures proximal and distal forelimb movements.[32,33] Animals are given several pieces of two differently shaped cereal (doughnut and spherical) each day for one week prior to the test to acclimate them to the food. On the testing day, animals are placed

into a plexiglass cylinder and videotaped eating cereal pieces of both shapes. Videos are scored in slow-motion playback on a 10-point (0-9) scale with the highest scores of both pieces recorded for each animal.

Limb asymmetry was measured by observing the individual use of either the right or left arm in exploration.[65] Animals were placed into a plexiglass cylinder (20 cm height x 20 cm diameter) for 5 minutes to encourage exploratory behavior. During exploration, rats will rear and place their forepaws against the walls of the cylinder. Forelimb placements were scored by slow video-playback for contralateral, ipsilateral and simultaneous forelimb placements. Simultaneous placements were defined as both limbs contacting the wall of the cylinder within <0.5 sec.

Tissue processing and histology

At the completion of each experiment, all animals were sacrificed by injection of Fatal-Plus (Dearborn, MI) and perfused with saline (0.9% NaCl) followed by 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (pH 7.5). The brain and cervical spinal cord were promptly dissected and post-fixed in 4% PFA overnight at 4°C. Tissue samples were then transferred to 30% sucrose for an additional 3 days prior to sectioning. 30 μm serial tissue sections were taken either sagittally or coronally on a cryostat and placed into cyroprotectant (containing Na_2HPO_4 H_2O, NaH_2PO_4, sucrose, PVP-40 and ethylene glycol) for long-term storage at -20°C.

Immunofluorescence

For immunohistochemical analysis of specific proteins, every fifth free-floating section was permeabilized in 0.3% Triton X-100 with 5% normal donkey serum to block non-specific binding sites. Samples were then incubated in the following primary antibodies: rabbit-anti-DsRed (1:500; Clontech, #632496), mouse-anti-NeuN (1:100; Millipore #MAB377), goat-anti-choline acetyl transferase (ChAT) (1:50; Millipore #AB144P), guinea pig-anti-vGlut2 (1:500; Millipore #AB2251), rabbit-anti-PKCɣ (1:500; Abcam #ab71558) or mouse-anti-GFAP (1:500; Dako #20334) overnight at 4°C. The next day, samples were incubated with secondary antibodies conjugated to appropriate fluorophores (Jackson ImmunoResearch Laboratories, Inc.; West Grove, PA) and mounted and photographed using Zeiss Axiovision software (Carl Zeiss Microscopy, Thornwood, NY).

Confocal images were taken using a Leica SP8 3000 Microscope (Leica Microsystems, Buffalo Grove, IL) with lasers pre-tuned to 488 nm (eGFP), 552 nm (Cy3) and 638 nm (AlexaFluor 647). Z-stack of images were taken using a 40X oil-immersive objective (NA 1.25) with 3X zoom for visualization of pre-synaptic terminals. Representative single plane images of terminals (1024 x 1024 pixels; section thickness 20 μm) were taken using maximum intensity projection. Confocal images were acquired in sequential mode to avoid crosstalk between channels (488, 552 and 638 nm). Maximum intensity projection and orthogonal sectioning was generated using Leica LASX software (Buffalo Grove, IL).

BDA staining and quantification

For quantification of BDA-labeled CST fibers, sections containing the red nucleus or C3-C4 spinal cord were incubated with 3% H_2O_2 in methanol to block endogenous peroxidases. Samples were incubated for 1 hr at room temperature using Vectastain Elite ABC reagents (Vector Laboratories, Burlingame, CA) and then developed using the peroxidase substrate 3'3'-diaminobenzidine (DAB) (SigmaFAST #D4293, Sigma-Aldrich, St. Louis, MO). Sections were mounted and subsequently dehydrated in ascending alcohol concentrations and coverslipped with permount. Mounted sections were imaged using Stereoinvestigator software (Microbrightfield, Inc., Williston, VT) to obtain monochrome images. Monochrome images were analyzed for CST fiber density using Nikon Elements 3.0. In brief, a region of interest (ROI) was drawn around the red nucleus or C3-C4 grey matter respectively. Intensity of BDA signal was thresholded to background for each image and analyzed per μm^2.

Electrophysiology

Whole-cell patch clamping of cortical slices was performed to determine function of hM4Di. AAV2-TetOn and HiRet-TRE-hM4Di-mCherry was mixed in a 1:1 ratio and injected into the sensorimotor forelimb cortex of several animals. Cortical slices were prepared for whole-cell patch clamp recordings.[66,67] Rats were euthanized by rapid decapitation and brains were placed into ice-cold artificial cerebrospinal fluid (ACSF) containing sucrose (248 mM) in place of NaCl. Coronal slices with the sensorimotor forelimb cortex (250 mm thick) were obtained using a Vibratome 3000 Plus (Vibratome, Bannockburn, IL) and placed in ACSF ((in mM): 124 NaCl, 2.5 KCl, 2 NaH_2PO_4, 2.5 $CaCl_2$, 2 $MgSO_4$, 10 dextrose, and 26 $NaHCO_3$) at 35°C bubbled with 95% O_2/5% CO_2 for 1 hr prior to being maintained at room temperature.

Slices were transferred to a recording chamber (Warner Instruments, Hamden, CT) and continuously perfused with ACSF at 1.5-2.0 mL/min. Only one cell was recorded per brain slice. Neurons were visualized using a Nikon E600 upright microscope fitted with differential interference contrast and infrared filters and equipped for epi-fluorescence with EGFP/FITC/Cy2/AlexaFluor 488 and TRITC/Cy3 long pass filter kits (Optical Apparatus, Ardmore, PA). Resistance of recording electrode was 4-6 mOhm when filled with an intracellular solution (in mM): 70 Kglucoonate, 70 KCl, 2 NaCl, 4 EGTA, 10 HEPES, 4 MgATP, 0.3 Na_2GTP, 0.1% biocytin, pH 7.3.

Neuronal excitability was assessed in whole-cell recordings under current clamp conditions with a HEKA patch-clamp EPC-10 amplifier (HEKA Elecktronik Lambrecht/Pfalz, Germany). A series of currents (100 to 160 pA, step +20) were injected to each cell assess the excitability by recording voltage responses. Once stimulated neurons stabilized, CNO (10 μM) was applied to bath solution for 3 minutes and the series of stimulation currents were re-applied to determine changes of voltage responses. Then slices were washed for 15 minutes with regular ACSF, and currents were re-applied to assess neuronal excitability. Recorded tissue was post-

fixed in 4.0% paraformaldehyde for 20-24 h and kept in 20% sucrose solution for fluorescent immunohistochemical methods (Li and Kirby, 2016; Kirby et al., 2008) to visualize the recorded biocytin-filled cell to identify whether the recorded cells were mCherry^{+} neurons with hM4Di expression.

QUANTIFICATION AND STATISTICAL ANALYSIS

All statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, Inc., La Jolla, CA). Statistical evaluations of behavioral data in Figures 2, 3, 4, and S2 were analyzed by repeated measure ANOVA followed by a Sidak's post-hoc test for statistical significance against groups. All behavioral data is represented as mean ± SEM. All morphological analysis in Figure 7 were done using one-way ANOVA with Sidak's post-hoc and represented as mean ± SD. Statistical analyses of mean differences in Figure 4J were analyzed using t-test and represented as mean ± SEM. Details of statistic scores are found in the results section and figure legends.