Combination of chondroitinase ABC and AAV-NT3 promotes neural plasticity at descending spinal pathways after thoracic contusion in rats

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Hunanyan AS, Petrosyan HA, Alessi V, Arvanian VL. Combination of chondroitinase ABC and AAV-NT3 promotes neural plasticity at descending spinal pathways after thoracic contusion in rats. J Neurophysiol 110: 1782–1792, 2013. First published July 17, 2013; doi:10.1152/jn.00427.2013.—Transmission through descending pathways to lumbar motoneurons, although important for voluntary walking in humans and rats, has not been fully understood at the cellular level in contusion models. Major descending pathways innervating lumbar motoneurons include those at corticospinal tract (CST) and ventrolateral funiculus (VLF). We examined transmission and plasticity at synaptic pathways from dorsal (d)CST and VLF to individual motoneurons located in ventral horn and interneurons located in dorsomedial gray matter at lumbar segments after thoracic chronic contusion in adult anesthetized rats. To accomplish this, we used intracellular electrophysiological recordings and performed acute focal spinal lesions during the recordings. We directly demonstrate that after thoracic T10 chronic contusion the disrupted dCST axons spontaneously form new synaptic contacts with individual motoneurons, extending around the contusion cavity, through spared ventrolateral white matter. These detour synaptic connections are very weak, and strengthening these connections in order to improve function may be a target for therapeutic interventions after spinal cord injury (SCI). We found that degradation of scar-related chondroitin sulfate proteoglycans with the enzyme chondroitinase ABC (ChABC) combined with adeno-associated viral (AAV) vector-mediated prolonged delivery of neurotrophin NT-3 (AAV-NT3) strengthened these spontaneously formed connections in contused spinal cord. Moreover, ChABC/AAV-NT3 treatment induced the appearance of additional detour synaptic pathways innervating dorsomedial interneurons. Improved transmission in ChABC/AAV-NT3-treated animals was associated with increased immunoreactivity of 5-HT-positive fibers in lumbar dorsal and ventral horns. Improved locomotor function assessed with automated CatWalk highlights the physiological significance of these novel connections.

Contusions are the most common spinal cord injury (SCI) in humans and often result in partial damage or complete lesion of the corticospinal tract (CST), which is one of the major descending pathways contributing to the control of voluntary movement in mammals (Porter and Lemon 1993). Although CST anatomy in rodents differs from that in cats, monkeys, and humans, rat contusion models exhibit a striking similarity to most human spinal trauma (Metz et al. 2000; Noreenberg et al. 2004; Scheff et al. 2003). Anatomical experiments have revealed that after dorsal lesions the dorsal (d)CST has the capacity to spontaneously sprout through the gray matter toward propriospinal axons located in the lateral and ventrolateral white matter in rats (Bareyre et al. 2004; Hill et al. 2001; Onifer et al. 2011). Despite this reported ability of intrinsic spinal circuits spontaneously reorganizing anatomically after dorsal lesions, recovery of locomotor function after contusion injury is limited in rats and humans.

To mediate better functional recovery, it is important that 1) sprouted fibers make appropriate functional synaptic contacts with target neurons and 2) these synaptic connections are strong enough to mediate function. Effects of contusion SCI on synaptic function at neural pathways transmitting to individual neurons have not been examined yet. Here we used a single-cell electrophysiology approach to examine synaptic projections from dCST and ventrolateral funiculus (VLF) to the same interneurons and motoneurons in lumbar gray matter after thoracic contusion in adult rats. In an attempt to strengthen transmission in contused spinal cord, we used an additive treatment comprised of chondroitinase ABC (ChABC) and adeno-associated viral (AAV) vector-mediated prolonged delivery of neurotrophin NT-3 (AAV-NT3).

The choice of this dual treatment was based on the knowledge that accumulation of chondroitin sulfate proteoglycans (CSPGs; Dou and Levine 1994; Galtrey and Fawcett 2007; Silver and Miller 2004; Snow et al. 1990) and insufficient neurotrophin support (Arvanian 2013; Fortun et al. 2009; Grill et al. 1997; Mendell et al. 2001; Schnell et al. 1994) are major reported obstacles known to restrict better recovery after SCI. Degradation of CSPGs with the enzyme ChABC was shown to promote sprouting of fibers following cervical crush (Barratt et al. 2006; Bradbury et al. 2002), hemisection (Houle et al. 2006; Iseda et al. 2008), transection (Fouda et al. 2005), and contusion (Caggiano et al. 2005; Tom et al. 2009, but see Iseda et al. 2008) injuries. Treatment with ChABC combined with neurotrophin NT-3 induced greater effects on axonal sprouting following dorsal lesions (Lee et al. 2010; Massey et al. 2008).

In addition to promoting axonal sprouting, treatment with ChABC partially restored axonal conduction deficits resulting from the accumulation of CSPGs in chronically hemisected spinal cord (Hunanyan et al. 2010). Neurotrophin NT-3 administered alone via engineered fibroblasts strengthened transmission in noninjured spinal cord (Arvanian et al. 2003) but induced negligible effects on synaptic transmission and function after thoracic hemisection SCI (Schnell et al. 2011). Applied together, ChABC and NT-3 (via fibroblasts) were key components of a combination treatment that induced formation of novel detour connections around the lesion and improved...
locomotor function after lateral hemisection (García-Álías et al. 2011).

In this study we used contusion SCI, which is a more realistic model, and examined effects of injury and combination treatment with ChABC and NT-3 on synaptic projections from dCST and VLF to individual lumbar neurons. For clinically relevant delivery of NT-3 we used AAV-mediated gene transfer, reported as the most promising therapeutic transgene delivery system for prolonged, safe, and effective neurotrophin delivery (Blits et al. 2003; Boyce et al. 2012; Fortun et al. 2009). To evaluate the functional significance of physiological changes, we have compared results of intracellular recordings with anatomical plasticity and recovery of locomotor function.

Some results of this study have been published in abstract form (Hunanyan et al. 2012a).

**MATERIALS AND METHODS**

Experiments using adult (~210 g) female Sprague-Dawley rats were carried out in accordance with protocols approved by the Institutional Animal Care and Use Committees at SUNY Stony Brook and the Northport Veterans Affairs Medical Center. We used three animal groups to conduct electrophysiological recordings: 1) noninjured, 2) SCI and control treatment [penicillinase and AAV-gfp (green fluorescent protein)], and 3) SCI and treatment with ChABC and AAV-NT3-gfp. Animals from the two SCI groups were pretrained to cross the CatWalk runway and then received a moderate contusion SCI at T10 spinal level and corresponding treatments immediately after injury. Intracellular recordings from lumbar gray matter dorsomedial interneurons and ventral horn motoneurons were performed 9–11 wk after SCI, treatment administration, and behavioral testing.

**Spinal Cord Injury and Treatment Administration**

Animals were anesthetized with 3% isoflurane in 100% O2 in an induction chamber and then transferred to a facemask delivering 1.5% isoflurane in 100% O2 to maintain anesthesia during surgery. Petrolatum ophthalmic ointment (Dechra Veterinary Products) was applied to the eyes to prevent desiccation. Rats were placed on a water-circulated heating pad to maintain body temperature at 36.5–37°C. Before surgery, animals received a subcutaneous injection of buprenorphine (0.01 mg/kg) to reduce postoperative pain. Contusion injury was performed at T10 spinal level with a computer-controlled IH-0400 Impactor device (Precision System and Instrumentation) as isoflurane in 100% O2 to maintain anesthesia during surgery. Pertussis toxin (1 mg/kg intraperitoneal injections) followed by one-fifth of the initial dose administered intramuscularly throughout experiments when needed. Tracheotomy was performed to provide artificial ventilation if necessary. Heart rate and expired CO2 were monitored continuously, and body temperature was maintained at 36–37°C with an automated controlled heating pad. Two dorsal laminectomies of the spinal cord were performed to excise T8–T10 (for placement of the stimulation electrodes and for acute dorsal column or lateral hemisection lesions) and L1–L6 (for placement of the recording electrode). L1–L6 ventral spinal segments were fixed tightly between custom-made bars to prevent movement of the spinal cord during recordings. Intracellular (Axoclamp 900A amplifier, Molecular Devices) recordings were performed from L6 spinal segment with a sharp glass microelectrode (50–to 80-MΩ resistance, filled with 3 M K-acetate) attached to a hydraulic microdrive (David Kopf Instruments), which precisely (with accuracy to 1 μm) measures the depth of the tip of the electrode. The glass microelectrode was positioned perpendicular to the cord, between the midline and the dorsal root entry zone, and recordings were performed starting from dorsal surface. We recorded from interneurons located in dorsomedial gray matter (at depth 0.05–1.2 mm) and ventral horn motoneurons (at depth 1.3–2.3 mm) in each rat. All neurons were identified by their ability to generate action potentials in mm caudal to the injury epicenter at a depth of 1 mm. This AAV-rh10 serotype has been shown to result in extremely high transduction levels in mice brain (Cearley and Wolfe 2006), and our preliminary study demonstrates an excellent transduction of both neurons and glial cells in contused spinal cord of adult rats (Petrosyan et al. 2012). After the injuries, the muscles were sutured with 4-0 monocryl (Ethicon) and skin was closed with wound clips followed by subcutaneous injections of antibiotic (Baytril, 5 mg/kg) and 5 ml of sterile lactated Ringer solution. Injections of antibiotic, analgesic, and Ringer solution were administered for 3 days after injury.

**Confirmation of Treatment Delivery: ChABC-Induced Digestion of CSPGs and AAV-Mediated gfp Expression in Spinal Cord**

To demonstrate the effectiveness of CSPG digestion by ChABC, horizontal sections (T9–T11) of spinal cords were assessed 1 wk after injury in two animals from each SCI group, i.e., contusion/control (penicillinase + AAV-gfp) and contusion/treated (ChABC + AAV-NT3-gfp) groups. Immunostaining with 2B6 antibody specific for CSPG digestion (Mson et al. 2001) revealed that in control contused animals the immunoreactivity of 2B6 was sparse, while in ChABC-treated animals strong 2B6 immunoreactivity in the vicinity of injury was detected (Fig. 1, D and E).

In vivo Intracellular Recordings

There are four important features of in vivo intracellular recording: 1) the ability to record even very weak signals that are impossible to detect by alternative electrophysiological methods, 2) the ability to identify whether the recorded cell is a motoneuron by using an antidromically evoked action potential, 3) the ability to determine synaptic inputs to one individual neuron from different spinal tracts, and 4) the ability to study reorganization of the synaptic pathways induced by the SCI and the treatments with online acute focal spinal lesions.

**Recording procedure.** All electrophysiological recordings were performed 9–11 wk after injury. Rats were deeply anesthetized with a ketamine (80 mg/kg, 0.5 ml)-xylazine (10 mg/kg, 0.5 ml) mixture (intraperitoneal injections) followed by one-fifth of the initial dose administered intramuscularly throughout experiments when needed. Tracheotomy was performed to provide artificial ventilation if necessary. Heart rate and expired CO2 were monitored continuously, and body temperature was maintained at 36–37°C with an automated controlled heating pad. Two dorsal laminectomies of the spinal cord were performed to excise T8–T10 (for placement of the stimulation electrodes and for acute dorsal column or lateral hemisection lesions) and L1–L6 (for placement of the recording electrode). L1–L6 ventral spinal segments were fixed tightly between custom-made bars to prevent movement of the spinal cord during recordings. Intracellular (Axoclamp 900A amplifier, Molecular Devices) recordings were performed from L6 spinal segment with a sharp glass microelectrode (50–to 80-MΩ resistance, filled with 3 M K-acetate) attached to a hydraulic microdrive (David Kopf Instruments), which precisely (with accuracy to 1 μm) measures the depth of the tip of the electrode. The glass microelectrode was positioned perpendicular to the cord, between the midline and the dorsal root entry zone, and recordings were performed starting from dorsal surface. We recorded from interneurons located in dorsomedial gray matter (at depth 0.05–1.2 mm) and ventral horn motoneurons (at depth 1.3–2.3 mm) in each rat. All neurons were identified by their ability to generate action potentials in...
response to depolarizing current injection through the same recording electrode. Motoneurons were identified by their antidromic action potential to electrical stimulation of cut L5 ventral root. The resting membrane potential of neurons used for analysis ranged from $-55$ to $-65$ mV. Recordings were collected from 10–15 neurons in each rat. Maximum responses from each neuron (10–30 consecutive responses/cell) were averaged. These average values were compared over all animals, and for statistical analysis we used both the number of animals and the total number of cells in each group.

Electrical stimulation protocols. We examined synaptic responses of these neurons evoked by electric stimulation (70-μs duration, 1-Hz frequency; A300 Pulsemaster/A360 Stimulus Isolator, World Precision Instruments) of dCST and VLF at T6. For electric stimulation of dCST and VLF we used two identical tungsten electrodes inserted into spinal cord at the appropriate depth so that the positions were not changed throughout the experiment. In all experiments, electric stimulations were applied ipsilateral to recording. For stimulation of VLF, a tungsten electrode (resistance 300 KΩ; FHC, Bowdoin, ME) was positioned between the dorsal root entry zone (at an angle of $\sim 20^\circ$, tip directed caudally) and the lateral edge of the cord and lowered to a depth of $\sim 1.7$ mm (Arvanian et al. 2009). For electric stimulation of dCST, a second tungsten electrode was positioned 0.1 mm from dorsal midline (at an angle of $\sim 15^\circ$, tip directed caudally) and lowered to a depth of $\sim 1$ mm (Hunanyan et al. 2012b). The positions of stimulation and recording electrodes are schematically presented in Fig. 1.

Acute dorsal column lesion and lateral hemisection during intracellular recordings. To examine synaptic pathways through dCST and VLF, we performed an acute focal dorsal column lesion followed by a lateral hemisection at T8, i.e., between stimulating and recording electrodes, ipsilateral to the recording/stimulation side, respectively (see Fig. 1B). We performed an acute lesion of the dorsal column to interrupt the transmission through dorsal corticospinal fibers (described in Alstermark et al. 2004) and an acute lateral hemisection to interrupt transmission through lateral white matter (described in Schnell et al. 2011). Each acute lesion was performed after recording from several neurons in the same rat. During continuous recording from the “last” neuron (either a ventral horn motoneuron in some animals or a dorsomedial interneuron in other animals) we performed the acute lesion and examined the effect of the acute lesion on the responses evoked by stimulation of both dCST and VLF. Synaptic responses from both dCST and VLF were then measured from several additional interneurons and motoneurons.

Fig. 1. Corticospinal tract damage induced by thoracic contusion and confirmation of treatment delivery. A: horizontal section at the site of injury (at T10) taken at the level of the dorsal corticospinal tract (dCST) 10 wk after 150-kdyn contusion showing complete disruption of the dCST. B: diagram showing the positions of the stimulating electrodes in dCST and ventrolateral funiculus (VLF) at T6 and intracellular recording electrode at L5 in relation to T10 contusion (spinal cord injury [SCI]). C: diagram to identify area used to examine treatment delivery (boxed area just caudal to contusion epicenter at the level of dCST). D and E: immunoreactivity for 2B6, a marker of chondroitin sulfate proteoglycans (CSPGs) digested by chondroitinase ABC (ChABC), 1 wk after injury: absence of signal in control penicillinase (P'ase)-injected animals (D) and positive immunoreactivity of 2B6 in ChABC-injected spinal cord 1 wk after injury (E). F: T10 horizontal section 10 wk after injury, showing excellent transduction after AAV-green fluorescent protein (gfp) intraspinal injection: gfp-positive cells and axons close to injury epicenter. G: L5 cross section showing gfp-positive fibers in L5 spinal cord; numerous gfp-positive fibers (at arrows) crossing white/gray matter boundary (outlined) were observed. Scale bar, 100 μm.
Statistical Analysis

For statistical analyses we used SigmaPlot 11.0 software (Systat Software). Data were analyzed with a one-way ANOVA or a one-way ANOVA on ranks followed by Tukey’s post hoc or Dunn’s test. All data are presented as means ± SE, and P < 0.05 was considered statistically significant.

RESULTS

Synaptic Projections from Thoracic dCST and VLF to Lumbar Ventral Horn Motoneurons and Dorsomedial Interneurons in Noninjured Rat

Lumbar motoneurons, characterized by Sir Charles Sherrington as “the final common path” (Sherrington 1906), are known to innervate leg muscles, are synaptically connected with neurons in lumbar dorsomedial gray matter, and receive synaptic inputs from the descending pathways, including major pathways at CST and VLF (Lemon 2008). Despite well-characterized monosynaptic responses in lumbar motoneurons from ipsilateral VLF in adult rats (Arvanian et al. 2009) and responses from CST in noninjured primates and cats (Edgley et al. 1997; Jankowska et al. 1993), there is little physiological evidence regarding projections to lumbar motoneurons from dCST in rats (Bogatyrev and Shapovalov 1973). In addition, there is an absence of knowledge as to whether these two tracts may have physiologically meaningful synaptic projections onto the same neurons in adult rat spinal cord. To resolve this question, we recorded intracellularly from neurons located in dorsomedial gray matter (referred to as interneurons throughout the text) and motoneurons located in ventral horn (referred to as motoneurons) in each rat. In each recorded neuron, we examined responses evoked by electric stimulation of dCST and VLF at T6. To verify synaptic pathway we compared responses measured before and after acute focal lesions of the dorsal column, followed by lateral hemisection, between recording and stimulating electrodes.

Motoneurons receive many monosynaptic projections from VLF and few polysynaptic projections from dCST. Consistent with our previous study in adult noninjured rats (Arvanian et al. 2009), electric stimulation of VLF evoked monosynaptic EPSP in almost all recorded L5 ventral horn motoneurons. Stimulation of dCST, however, evoked responses only in ~44% of motoneurons recorded (Table 1). A noticeable difference between responses evoked by stimulation of VLF and dCST in the same motoneurons was that all VLF-evoked responses were monosynaptic (Fig. 2A, traces 1A), while dCST-evoked responses were polysynaptic (Fig. 2A, traces 2A). Evidence that motoneuron responses evoked by VLF are most probably monosynaptic is based on short latency, steep rising phase, and negligible fluctuation in both latency and amplitude of these responses (amplitude 5 ± 0.5 mV, latency 1.4 ± 0.19 ms; n = 7 rats, 73 cells) (Fig. 2A, traces 1A) as previously discussed (Arvanian et al. 2009). The following results suggest that motoneuron responses from dCST are most probably polysynaptic: 1) these responses had smaller amplitude and longer latency (amplitude 2.9 ± 0.3 mV, latency 3.6 ± 0.4 ms; n = 7 rats, 57 cells; P < 0.05; Fig. 2A, traces 2A) compared with VLF-evoked EPSPs in the same motoneurons, and 2) EPSPs evoked in the motoneurons after electric stimulation of dCST displayed a less steep rising phase and were variable in shape and peak deflection compared with abrupt VLF-evoked responses in the same motoneuron. The major difference between dCST- and VLF-evoked motoneuron responses was their sensitivity to acute lesions of the dorsal column. Acute transection of the dorsal column, between recording and stimulating electrodes, resulted in elimination of dCST-evoked responses in motoneurons (Fig. 2A, traces 2B) but VLF-evoked responses of the same motoneuron were sustained (Fig. 2A, traces 1B). The VLF-evoked responses in motoneurons that were sustained after dorsal column lesion were
abolished after further ipsilateral hemisection (not shown). These results strongly suggest that in noninjured spinal cord the same motoneurons in lumbar ventral horn may receive monosynaptic inputs from VLF and polysynaptic inputs from dCST; moreover, motoneuron responses from these two inputs are realized through activation of two independent synaptic pathways.

Interneurons do not receive functional projections from VLF but receive both monosynaptic and polysynaptic projections from dCST. Electric stimulation of VLF (which evoked monosynaptic responses in ventral horn motoneurons; Fig. 2A, traces 1a) did not induce measurable synaptic responses in dorsomedial interneurons in the same animal (Fig. 2A, traces 3a). Stimulation of dCST, however, evoked either monosynaptic (shorter latency of 1.6 ± 0.2 ms, ~30% of cells recorded) or polysynaptic (longer latency of 3.1 ± 0.3 ms, ~70% cells) EPSPs (amplitude 2.5 ± 0.4 mV; Fig. 2A, traces 4a, n = 7 rats, 57 cells) in these interneurons. After acute lesion of the dorsal column all (monosynaptic and polysynaptic) EPSPs of dorsomedial interneurons evoked from dCST were completely abolished in these noninjured animals (Fig. 2A, traces 4b). These results suggest that in noninjured spinal cord dCST has functional synaptic connections with dorsomedial lumbar interneurons, while VLF does not.

Perturbation of Synaptic Projections After Chronic Thoracic Contusion in Control Treated Rats (Penicillinase and AAV-gfp)

Cresyl violet staining of horizontal sections revealed that chronic midthoracic moderate contusion injury induced complete disruption of dCST at injury epicenter (Fig. 1A).

Motoneurons: projections from VLF sustained but weakened; projections from dCST through dorsal column abolished but new weak synaptic connections from dCST through ventrolateral white matter to lumbar motoneurons formed spontaneously. After chronic thoracic contusion injury, stimulation of VLF evoked weak but still measurable EPSPs in ventral horn motoneurons (1.4 ± 0.18 mV, n = 6 rats, 47 cells) and longer latency (2.4 ± 0.2 ms) compared with uninjured animals (Fig. 2B, traces 1a). Acute lesion of the dorsal column at T8 did not alter these responses (Fig. 2B, traces 1b). These results suggest that after contusion SCI projections from VLF to ventral horn motoneurons were sustained, although these responses were dramatically attenuated.

Stimulation of dCST evoked small-amplitude (1.2 ± 0.09 mV) polysynaptic EPSP responses in motoneurons, and these responses appeared to be de novo (see Fig. 2B, traces 2a; Table 1). A striking result was that in contused spinal cords these dCST-evoked responses were sustained after acute lesions of T8 dorsal column (Fig. 2B, traces 2b); these responses were abolished after further ipsilateral hemisection (not shown). Note that corresponding dCST-evoked responses in uninjured cord were abolished after similar acute lesion of the dorsal column (Fig. 2A, traces 2b). These results strongly suggest that after chronic midthoracic contusion injury dCST fibers spontaneously make a small number of new functional synaptic connections around the contusion cavity, through spared ventrolateral white matter, to lumbar L5 motoneurons.

Interneurons: still do not receive projections from VLF and lose all projections from dCST. Recordings from dorsomedial interneurons revealed that responses from VLF (which were not evident in noninjured rats; Fig. 2A, traces 3a) were still lacking in contused animals (Fig. 2B, traces 3a). Functional synaptic inputs from dCST to interneurons (which were present in noninjured rats; Fig. 2A, traces 4a) were completely abolished in contused rats (Fig. 2B, traces 4a). These results demonstrate that dorsomedial interneurons completely lost all functional projections from dCST after contusion SCI.

Table 1. Summary of results of electrophysiological experiments

<table>
<thead>
<tr>
<th>Electric Stimulation at T8</th>
<th>EPSPs Recorded from DM Interneurons</th>
<th>n</th>
<th>EPSPs Recorded from VH Motoneurons</th>
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<tr>
<td></td>
<td>Noninjured</td>
<td></td>
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<tr>
<td>dCST</td>
<td>2.5 ± 0.4 mV/3.1 ± 0.3 ms/55%</td>
<td>6</td>
<td>2.9 ± 0.3 mV/3.6 ± 0.4 ms/44%</td>
<td>7</td>
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<tr>
<td>VLF</td>
<td>0 mV/0 ms/0%</td>
<td></td>
<td>5 ± 0.5 mV/1.4 ± 0.19 ms/99%</td>
<td>7</td>
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<tr>
<td>Contusion, control group (P'ase + AAV-gfp)</td>
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<tr>
<td>dCST</td>
<td>0 mV/0 ms/0%</td>
<td></td>
<td>1.2 ± 0.09 mV/4.2 ± 0.1 ms/15%</td>
<td>6</td>
</tr>
<tr>
<td>VLF</td>
<td>0 mV/0 ms/0%</td>
<td></td>
<td>1.4 ± 0.18 mV/2.4 ± 0.2 ms/65%</td>
<td>6</td>
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<tr>
<td>Contusion, treatment group (ChABC + AAV-NT3-gfp)</td>
<td></td>
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<tr>
<td>dCST</td>
<td>1.5 ± 0.2 mV/3.2 ± 0.5 ms/36%</td>
<td>7</td>
<td>1.8 ± 0.15 mV/4.1 ± 0.4 ms/43%</td>
<td>7</td>
</tr>
<tr>
<td>VLF</td>
<td>1.7 ± 0.3 mV/2.9 ± 0.6 ms/40%</td>
<td>7</td>
<td>2.2 ± 0.2 mV/2.6 ± 0.4 ms/80%</td>
<td>7</td>
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</table>

Values, expressed as amplitude/latency/% of neurons responding to stimulation of either dorsal corticospinal tract (dCST) or ventrolateral funiculus (VLF) vs. total number of recorded neurons, are means ± SE for n animals (see text for total number of neurons recorded in each case). To evoke excitatory postsynaptic potential (EPSP) responses, electrical stimulation of VLF or dCST at T8 spinal level was applied. Recordings were performed at L5, dorsomedial (DM) gray matter interneurons and ventral horn (VH) motoneurons. ChABC, chondroitinase ABC; AAV-NT3, adeno-associated viral vector-mediated prolonged delivery of neurotrophin NT-3; P'ase, penicillinase.

Treatment with ChABC Combined with AAV-NT3 Strengthened Connections in Damaged Spinal Cord and Encouraged Establishment of Novel Detour Synaptic Connections Around Contusion Cavity

Motoneurons: treatment strengthened survived projections from VLF and detour projections from dCST. In rats that received contusion SCI and ChABC/AAV-NT3 treatment, EPSPs recorded from motoneurons and evoked by stimulation of both VLF and dCST displayed larger amplitude compared with contusion/control rats (VLF 2.2 ± 0.2 mV, dCST 1.8 ± 0.15 mV; n = 7 rats, P < 0.05; Fig. 2C, traces 1a and 2a). Consistent with the results obtained after acute lesions in contusion/control rats (Fig. 2B, traces 1b and 2b), motoneuron responses evoked by electric stimulation of either VLF or dCST remained unchanged after acute lesions of the dorsal column in contused ChABC/AAV-NT3 treated rats (Fig. 2C, traces 4a, 4b, 4c).
traces 1b and 2b). These results suggest that both survived projections from VLF and spontaneously formed detour connections from dCST through ventrolateral white matter to motoneurons were strengthened in contused cords that received ChABC/AAV-NT3 treatment.

Interneurons: novel detour projections from both VLF and dCST were established. Unexpectedly, our results indicated the appearance of entirely novel synaptic inputs from VLF to dorsomedial interneurons. In contused ChABC/AAV-NT3-treated animals, stimulation of VLF evoked polysynaptic responses in the dorsomedial interneurons (amplitude 1.5 ± 0.2 mV; Fig. 2C, traces 3b). Note that synaptic responses of dorsomedial interneurons from VLF were absent in contusion/control rats (Fig. 2B, traces 3a) and even in noninjured rats (Fig. 2A, traces 3a).

Synaptic responses of dorsomedial interneurons from dCST [which were present in noninjured rats (Fig. 2A, traces 4a) and absent in contused/control rats (Fig. 2B, traces 4a)] reappeared in contused treated cords (amplitude 1.5 ± 0.2 mV; Fig. 2C, traces 4b).

Fig. 2. Rearrangement of synaptic circuits after chronic midthoracic contusion: consecutive traces of excitatory postsynaptic potentials (EPSPs) evoked in L5 ventral horn (VH) motoneurons and dorsomedial (DM) interneurons from T6 ipsilateral dCST (red traces) and VLF (blue traces). Left: diagrams illustrating the positions of stimulating and recording electrodes in relation to chronic T10 contusion. A: noninjured rat. Traces 1a–4a: monosynaptic responses of VH motoneuron from VLF (1a); same VH motoneuron received polysynaptic inputs from dCST (2a); DM interneurons in the same rat did not show measurable synaptic responses from VLF (3a); same interneuron as in 3a received polysynaptic inputs from dCST (4a). Traces 1b–4b: corresponding responses recorded in same animal after acute lesion of the dorsal column at T8, i.e., between stimulation and recording electrodes. B: contusion SCI rat received control penicillinase and AAV-gfp treatment. C: contusion SCI rat received ChABC and AAV-NT3 treatment. D: diagrams illustrate the possible synaptic projections to lumbar DM interneurons and VH motoneurons from dCST (red lines) and VLF (blue lines) in noninjured rats (A), chronic contused rats receiving control treatment (B), and chronic contused rats receiving ChABC and AAV-NT3 treatment (C).
ChABC/AAV-NT3 treated animals (143 pool, around motoneurons, also was increased significantly in group. 5-HT immunoreactivity in the ventral horn motoneuron, 24%; Fig. 3 significantly in contused/treated (ChABC/AAV-NT3) rats (198 5-HT-positive fibers in lumbar dorsal horn increased significantly in SCI rats that received control (A) or ChABC + NT3 (B) treatment. 5-HT immunoreactivity in both dorsal and ventral horn increased in ChABC + NT3-treated rats. C and D: higher-magnification images of the ventral horn (area outlined by a solid-line box in A) taken from sections displayed in A and B, respectively, show an increase in the number of 5-HT-positive fibers in the vicinity of the motoneuron pool in ChABC + NT3-treated cord. E: summary of quantitative analyses of thresholded pixel area of 5-HT-positive fibers confirmed a significant increase in 5-HT-positive fibers in dorsal and ventral horn in ChABC + NT3 group. Error bars show SE. *P < 0.05. F: high-power image demonstrating presence of 5-HT-positive fibers in close association with neurons (most probably motoneurons, based on their size and morphology) in ventral horn. Areas outlined by dashed- and solid-line boxes in A denote regions selected for quantification in dorsal and ventral horn, respectively. Scale bars, 50 μm.

Effect of ChABC/AAV-NT3 Treatment on Locomotor Recovery

To assess locomotor performance after midthoracic contusion injury, rats were evaluated with the automated CatWalk device and gait parameters were compared between groups. All rats were pretrained and examined on CatWalk prior to surgery and then at 2-wk intervals until terminal electrophysiological recording experiments were carried out.

During the first 2 wk after injury rats did not show detectable hindlimb footprints; thus CatWalk assessment was started 2 wk after injury. The base of support, i.e., distance between hindlimbs, and regularity index, i.e., measurement of forelimb-hindlimb coordination at this time point, were not significantly different between SCI rats that received either control or ChABC/AAV-NT3 treatment (P > 0.05; Fig. 4).

However, beginning at 4 wk after injury the ChABC/AAV-NT3 group animals showed significantly decreased base of support compared with control treated rats (35.1 ± 0.9 mm for contused/treated vs. 39.1 ± 1.38 mm for contused/control groups, P < 0.05). Regularity index was significantly higher in ChABC/AAV-NT3-treated rats, showing that treated rats had better forelimb-hindlimb coordination compared with control treated rats (Fig. 4; P < 0.05). The ChABC/AAV-NT3-treated group showed significant improvement of locomotor function, i.e., narrower base of support and higher regularity index, compared with the control treated group at all remaining time points (Fig. 4).

DISCUSSION

Using in vivo single-cell electrophysiology, we demonstrate that in the intact spinal cord individual interneurons in dorso-
medial lumbar segments receive synaptic inputs from dCST and not from VLF, while ventral horn motoneurons receive inputs from both dCST and VLF. After contusion SCI, we detected the formation of weak detour synaptic pathways around the lesion cavity through the spared ventrolateral white matter to lumbar motoneurons. An important finding was that ChABC/AAV-NT3 dual treatment strengthened these spontaneously formed connections to motoneurons and, moreover, induced appearance of additional detour synaptic pathways around the disrupted dorsal column to dorsomedial interneurons.

**Independent Synaptic Projections from dCST and VLF to Same Lumbar Interneurons and Motoneurons in Noninjured Spinal Cord**

Both CST (Carmel et al. 2010; Hendriks et al. 2006; Martin 2005) and VLF (Hendriks et al. 2006; Loy et al. 2002; Reed et al. 2009; Schucht et al. 2002) are important spinal pathways for maintaining voluntary locomotion in humans and rats. Synaptic projections from VLF to lumbar motoneurons have been well characterized electrophysiologically in neonatal (Arvanov et al. 2000; Pinco and Lev-Tov 1994) and adult (Arvanian et al. 2009) rats. Transmission through CST to motoneurons has been examined mainly in cats and primates (Edgley et al. 1997; Jankowska et al. 1993). There are, however, only a few intracellular electrophysiological studies that have examined transmission from dCST to motoneurons in rats. These include intracellular recordings from cervical forelimb motoneurons (Alstermark et al. 2004; Babalian et al. 1993) and one report that described polysynaptic transmission from motor cortex to lumbar ventral horn motoneurons (Bogatireva and Schapovalov 1973) in noninjured rats. The present study provides the first intracellular examination of transmission from both dCST and VLF to the same dorsomedial interneurons and ventral motoneurons in lumbar spinal cord.

Consistent with our previous study in noninjured adult rats (Arvanian et al. 2009), midthoracic VLF projects monosynaptically to L₅ ventral horn motoneurons (Fig. 2A, traces 1a). However, synaptic transmission from VLF to L₅ dorsomedial interneurons was absent in the same animal (Fig. 2A, traces 3a). These electrophysiological results are consistent with the anatomical tracing experiment demonstrating that in noninjured adult rats midthoracic VLF axons project to L₅ gray matter mostly within the lumbar ventral, but not dorsal, gray matter (Reed et al. 2009). Note that because of the difficulty of intracellular recording from the small-sized interneurons, we do not exclude a possibility of some projections to the dorsomedial interneurons, which could not be detected.

Our results revealed that dCST makes synaptic contacts with both ventral horn motoneurons (Fig. 2A, traces 2a) and dorsomedial interneurons (Fig. 2A, traces 4a). Unlike monosynaptic responses evoked from VLF detected in almost all motoneurons, the responses of the same motoneurons to stimulation of dCST were polysynaptic and detected in only ~44% of lumbar motoneurons. Consistent with our later results, electrophysiological studies of Alstermark et al. (2004) have shown a lack of monosynaptic corticomotoneuronal connections at cervical levels in rats.

Another important difference between motoneuron monosynaptic responses from VLF and polysynaptic responses from dCST was that acute lesions of the dorsal column between stimulation and recording electrodes completely eliminated all postsynaptic responses from dCST, but responses from VLF in the same motoneurons were sustained (Fig. 2A, traces 4b and 1b). These results suggest that in noninjured spinal cord transmission from dCST to lumbar motoneurons is apparently realized through synaptic activation of the dorsomedial interneurons, which in turn are synthetically connected to ventral horn motoneurons. Consistent with this view, electron microscopy studies revealed no evidence for direct corticomotoneuronal synapses in the rat spinal cord (Yang and Lemon 2003), and recordings from rat spinal cord slices suggest that corticospinal transmission to ventral horn motoneurons most probably...
occurs through excitation of dorsal horn neurons at segmental level (Hori et al. 2002).

In conclusion, synaptic pathways from VLF and dCST to lumbar motoneurons are apparently independent, and the synaptic pathway from dCST to ventral horn motoneurons does not merge with VLF in noninjured spinal cord.

**Plasticity of Synaptic Connections After Thoracic Contusion**

Sprouting of lesioned CST axons is known to occur spontaneously rostral to spinal contusion (Hill et al. 2001; Steeneken et al. 2009) and dorsal lesions (Fouad et al. 2001; Weidner et al. 2001). On the basis of the results of anatomical tracing and intramuscular recordings of EMGs, it was hypothesized that sprouted dCST axons can make connections with cervical propriospinal neurons projecting to caudal spinal segments spanning the injury epicenter after thoracic dorsal hemisection in adult rats (Bareyre et al. 2004; Ghosh et al. 2010). Consistent with the proposed role of propriospinal projections as a major compensatory circuit in damaged spinal cord, locomotor deficits after thoracic contusion were found to be proportional to the amount of damage to the spared lateral and ventrolateral white matter (Bresnahan et al. 1991; Cao et al. 2005).

Our present electrophysiological study supports this view and provides new direct evidence that these spontaneously formed detour pathways from disrupted dCST through ventrolateral white matter can mediate weak functional synaptic contacts with individual ventral horn motoneurons in lumbar segments after thoracic contusion. We found that after moderate contusion that disrupts CST at dorsal column (Fig. 1) synaptic transmission from dCST to dorsomedial interneurons was abolished (Fig. 2B, traces 4a); transmission from VLF to lumbar ventral horn motoneurons was sustained but dramatically declined (Fig. 2B, traces 1a). The latter results are consistent with recent reports demonstrating conduction deficits in axons spanning the contusion epicenter in rats (Arvanian et al. 2006; James et al. 2011). An exciting result was the appearance of novel, although weak, detour connections from dCST to ventral horn motoneurons in contusion/control animals (Fig. 2B, traces 2a).

**ChABC/AAV-NT3 Treatment**

Treatment with ChABC alone, at upper CNS levels, was shown to improve function in some models of neurological deficiency, such as unilateral pyramidalotomy in mice (Starkey et al. 2012) and stroke in elderly rats (Soleman et al. 2012), although it did not induce neurological recovery in rabbit pups with intraventricular hemorrhage (Vinukonda et al. 2013). ChABC was found to improve function after dorsal lesions of cervical spinal cord (Bradbury et al. 2002). In cases of other SCI models, treatment with ChABC alone improved axonal sprouting, although it induced little or no recovery of locomotor function after hemisection (Allalain et al. 2011; Hunanyan et al. 2010) and contusion (Harris et al. 2010; Mountney et al. 2013; Tom et al. 2009).

The AAV-vector-mediated transgene delivery of neurotrophins [NT-3 and brain-derived neurotrophic factor (BDNF)] was shown to partially reverse chronic pain after SCI (Eaton et al. 2002) and rescue atrophy of rubrospinal neurons (Ruitenberg et al. 2004) and motoneurons (Blits et al. 2004) but induced only minor improvement of locomotor function in models of incomplete SCI (Blits et al. 2003; Fortun et al. 2009) and after complete transection of the spinal cord (Boyce et al. 2012).

Unfortunately, despite apparent progress, as demonstrated by anatomical and immunohistochemical studies, the therapeutic ability of neurotrophins and ChABC, applied separately, to promote recovery of functional loss after SCI is still limited. At present, multiple studies point to the necessity of combining delivery of neurotrophins with other treatments including ChABC (García-Alías et al. 2011; Lee et al. 2010; Massey et al. 2008) for superior improvements in plasticity and functional outcomes after spinal injury.

The major exciting finding of the present study was that combined administration of ChABC and AAV-NT3 induced strengthening of the weak connections to ventral motoneurons and establishment of novel functional synaptic connections from both VLF and dCST to the dorsomedial interneurons after thoracic contusion (Fig. 2C). Appearance of these novel synaptic connections was associated with increased density of 5-HT-positive fibers in lumbar ventral and dorsal segments (Fig. 3) and better locomotor function (Fig. 4). The improved plasticity of serotoninergic axons that coincided with improved transmission and locomotor function reported here is consistent with ample literature implicating the role of serotonergic descending pathways in somatosensory functions in the spinal cord and their role in motor function through proprioceptive feedback (Jankowska et al. 1997, 2000; Oatway et al. 2005; Schmidt and Jordan 2000).

As mentioned above, ventral motoneurons, known to innervate leg muscles, receive polysynaptic inputs from dCST most probably through excitation of the segmental dorsomedial neurons. Thus the strengthening of the weak connections to ventral motoneurons and the establishment of novel detour pathways from the disrupted CST to dorsomedial neurons in contused and ChABC/AAV-NT3-treated spinal cord reported here may have functional implications. These detour connections may effectively be targeted in further searches for treatment for more complete recovery of deficits in locomotion after spinal cord contusion injuries.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the author(s).

**AUTHOR CONTRIBUTIONS**

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