AWARD NUMBER: W81XWH-15-1-0595

TITLE: Enhancing Propriospinal Relays to Improve Functional Recovery after SCI

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REPORT DATE: October 2016

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2016	Annual	15 Sep 2015 - 14 Sep 2016

4. TITLE AND SUBTITLE
Enhancing Propriospinal Relays to Improve Functional Recovery after SCI

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-15-1-0595

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

This is the first annual report for the grant to enhance propriospinal relays to bypass a contusion injury to the spinal cord. We have completed experiments for subtask 1 – 4 of specific aim 1 and analysis of data for subtasks 2. We are still analyzing data for subtasks 3 – 4 which will be complete by the end of the year. Our data shows spontaneous recovery might be due to the preservation of several pathways including the pontine and medullary reticulospinal and the cervical propriospinal tracts. These are the primary neuronal population with surviving axons bypassing the lesion after a moderate contusion injury to the T10 region. This information will allow us to determine in plasticity rostral to the injury site participates in the recovery. However, corticospinal axons could sprouting into either the pontine or medullary reticular formation in addition to the cervical spinal cord. We are presently analyzing connectivity of other supraspinal pathways onto the cervical propriospinal neurons. The slight delay in analysis will not delay the overall experimental goals and we are proceeding to begin silencing experiments to determine the contribution of the surviving pathways to functional recovery. We are also planning to start pilot studies to examine regeneration and sprouting mediated by inhibiting PTEN using our peptides.

15. SUBJECT TERMS
Nothing listed

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	8	19b. TELEPHONE NUMBER *(include area code)*

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1. Introduction:

Spinal cord injury causes life-long neurological impairment, with loss of sensory and motor function distal to the point of injury. Despite the clinical impact of traumatic spinal cord injury (SCI), there is no effective treatment for SCI. As a consequence, there are approximately 400,000 patients with SCI in the United States. Experimental therapies to treat SCI have focused on approaches that promote neural regeneration, but major problems remain in achieving long distance regeneration of higher functioning motor control systems, such as the corticospinal tract, making restoration of voluntary locomotor control difficult. In many animal models, spontaneous recovery is often observed after incomplete injuries, leading to partial recovery over time. Spontaneous recovery is thought to be mediated by a number of repair mechanisms including recovery from spinal shock, sprouting and remyelination. Of these processes, sprouting of axons onto interneurons can form adaptive pathways relaying motor information past the lesion to spinal motor neurons driving locomotor responses caudal to the lesion. Indeed several studies using unilateral hemisection model in rodents demonstrate the importance of these relays in supporting spontaneous return of weight support or kinematic patterning of hind limb movements. In several of those studies lesioned supraspinal axons undergo collateral sprouting onto propriospinal interneurons bypassing the lesion. These propriospinal neurons synapse directly onto spinal motor neurons caudal to the lesion site driving locomotor responses. Propriospinal neurons can either span short distances up to six spinal segments or long distances interconnecting cervical and lumbar region. Of particular interest to the return of motor function are the descending propriospinal neurons. The vast majority of these neurons are involved in motor responses and localized to the intermediate zone of the spinal gray matter; an area when stimulated shows locomotor responses. These propriospinal neurons (PN) can either span short distances from the Thoracic to the lumbar region (TPN) or long distances interconnecting cervical and lumbar region. One role of Long-Descending Propriospinal Neuron (LDPN) tracts is to coordinate rhythmic movements of arms and legs during walking. In the cervical cord, they can receive direct supraspinal information from corticospinal, rubrospinal, reticulospinal and tectospinal axons. The majority of these propriospinal axons descend within the medio- and ventro-lateral funiculus entering the ventral horn to form multiple synaptic contacts directly onto motor neurons. After a moderate contusion injury, much of the lateral funiculus is preserves and many PNs are uninjured. The shorter forms of propriospinal neurons are thought to integrate this information, establishing synergistic ensembles to organize movement. Whether or not they contribute to functional recovery after contusive injury has never been determined, and whether or not TPN or LDPNs are more influential in recovery also remains unclear.

2. Keywords:

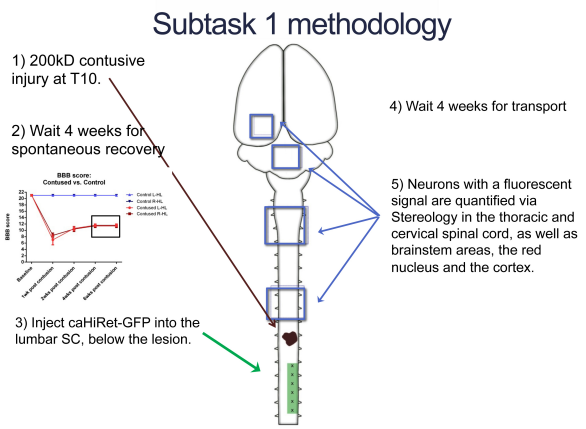
Propriospinal neurons, spinal cord injury, axon sprouting, spinal cord circuits, locomotion, functional recovery.

3. Accomplishments:

Subtask 1: Characterize the numbers and distribution of supraspinal and propriospinal neurons with axons bypassing the lesion and terminating in the lumbar spinal cord.

Although propriospinal axons are thought to be important for spontaneous return of hind limb locomotor function after unilateral thoracic hemisection, little is known of how these neurons affect hindlimb recovery after thoracic contusion injury. Within the first 4 weeks after either a mild or moderate contusion injury, animals show a progressive increase in functional recovery, plateauing approximately 4-6 weeks after injury. Moderate contusion injury typically spares part of the lateral and

ventrolateral funniculi. These regions are known to contain propriospinal and several supraspinal tracts, such as reticulospinal, ventral corticospinal, vestibulospinal axons, and in more dorsal regions rubrospinal axons. However, which population account for the functional recovery is presently unknown since contusion injury does not completely remove supraspinal axons. For the first subtask the aim was to characterize the location and numbers of neurons with axons surviving a T10 moderate contusive injury in relationship to those innervating the lumbar in non-injured rats. Figure 1 shows the general method used to map the neurons connecting to the lumbar spinal cord. Moderate contusion injury was made at the T10 level using an Infinite Horizon impactor with an impact force of 200 kDyn. Four weeks after injury, the point at which the recovery levels for the majority of animals plateau, we injected HiRet/GFP/Lentivirus into the lumbar spinal cord. This lentivirus uses the Rabies-G envelope protein for high efficiency retrograde transport, even after injury. It also contains a constitutively active promoter for expression of GFP for labeling of multiple neuronal populations with axons bypassing the lesion. Eight injection were made into the intermediate gray matter (lamina VII)/ventral horn region extending from L1 – L4. Four week post-injection animals were perfused, cryoprotected and sectioned for identification of GFP positive neurons in the cortex, red nucleus, medial/ventral medullary reticular formation, pontine reticular formation, C4-C7 and T5-T9 spinal cord. All GFP neuronal counts were done using standard stereological methods using Stereo Investigator software (MBF Bioscience) and a Leica M6000B, epifluorescent microscope.



Four weeks after injections of HiRet/GFP/lentivirus into the lumbar spinal cord some GFP labeled neurons within brainstem nuclei were observed (Figure 2). Brightly fluorescent neurons could be identified within the Red nucleus, pontine reticular formation and medullary reticular formation, but not within the motor cortex, locus coeruleus or vestibular nuclei. In general, the number of GFP labeled neurons within the red nucleus was approximately a tenth of those labeled after injection of HiRet/GFP into the cervical spinal cord, indicating that this pathway mostlikely contributes more to forelimb than hindlimb movement. Neuronal counts using unbiased stereological imaging showed that within the brainstem the majority of neurons innervating the intermediate zone within the lumbar

Figure 2

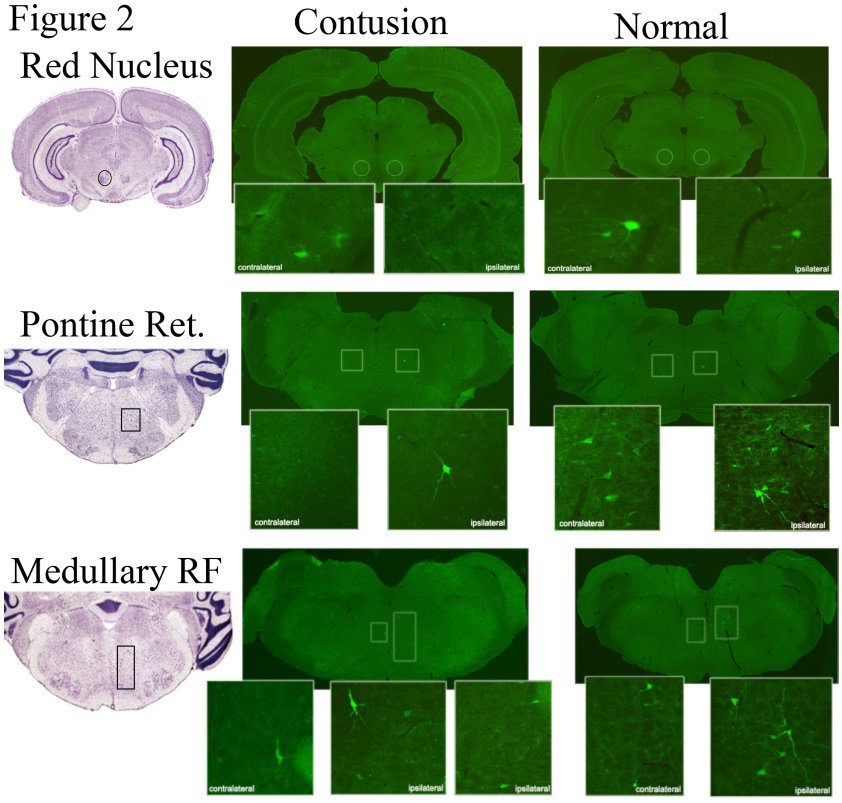
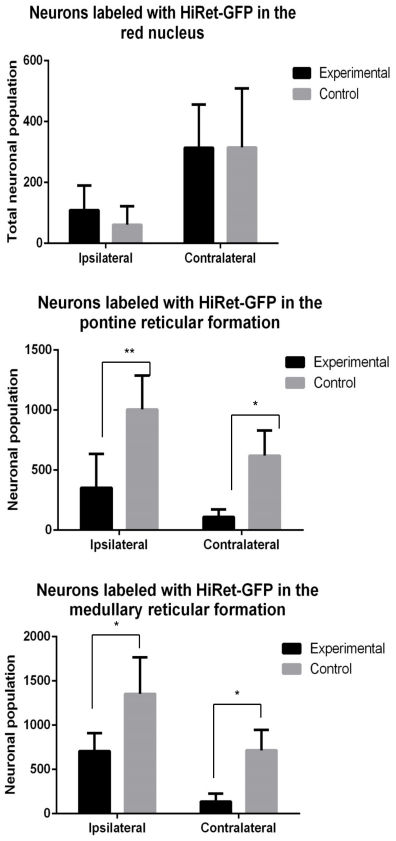


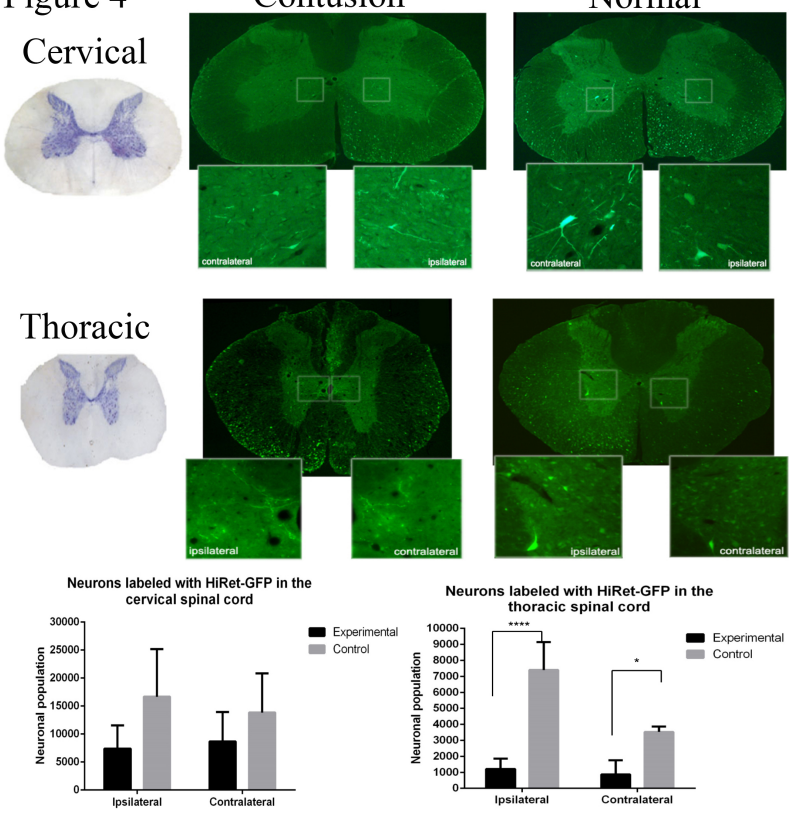
Figure 3



spinal cord originated from either the pontine or medullary reticular formation (Figure 3). These neurons are known to mediate locomotion and their preservation could contribute to locomotor recovery after injury. Analysis 8 weeks after a mid-thoracic contusion showed a significant decrease in the number of neurons labeled within either the pontine or medullary reticular formation. Eventhough, there was a significant loss of approximately 40% of the pontine and 55% of the medullary neurons retained connections within the injured lumbar spinal cord.

In these studies we observed the highest number of GFP labeled neurons originating within the spinal cord, particularly the cervical spinal cord (Figure 4). These propriospinal neurons are thought to connect spinal cord segments to support intersegmental communication. They are also thought to be important for locomotor recovery after spinal cord hemisection. These neurons show both ipsilateral and contralateral neuronal labeling. Interestingly, examination of the sections shows a very high density of axon labeling within the ventral and ventrolateral funiculi of the spinal cord. This is the region that will be the least damaged by a contusion injury and axons within this region have the highest probability to contribute to functional recovery. After injury there is a significant loss in thoracic propriospinal neurons (>75%), however, no statistical difference in the loss of cervical propriospinal neurons was observed, eventhough there was about a 50% loss in labeling. This could be due to there only being 4 animals/group. More animals will be examined to determine if this loss is significant.

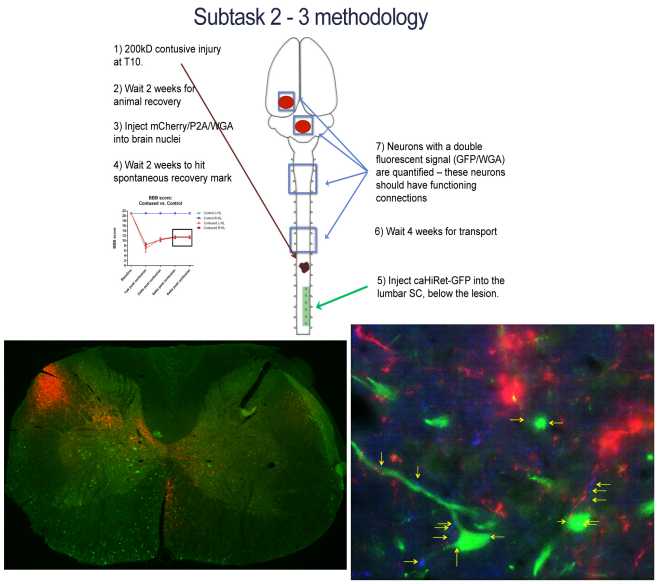
Figure 4



Subtask 2 and 3: Map connections of supraspinal axons onto propriospinal neurons (S2) and propriospinal axons onto lumbar neurons after contusion injury (S3).

To investigate the connections between supraspinal axons and LDPNs or TPNs, we injected mCherry/WGA/AAV into either the red nucleus, medullary reticular formation, or the pontine reticular formation (Figure 5). In general the experiments were done as described in S1 with the addition of mCherry/WGA/AAV injections. Low magnification images show labeling of the axons from the red nucleus (red) and cervical propriospinal neurons (green) labeled from injections

Figure 5



into the lumbar cord. Arrows show labeled neurons (blue) surrounded by numerous mCherry positive rubrospinal axons, many of which appear to have synaptic profiles associated with them. These experiments have been finished and the majority of sections cut and stained, however analysis is not complete due to several factors. The first being a minor problem with the StereoInvestigator software integration with the microscope, however this has been fixed. The present problem is the backlog of users who need to use the stereological system. Presently we have analyzed several animals in each group (n= 1-2/group) and have not observed much of a difference in the numbers of co-labeled cervical propriospinal neurons between normal and contusion injury, but in reality the number of spinal cord analyzed is too small to make that conclusion.

Subtask 4: Examine the functional contribution of either supraspinal or propriospinal neurons terminating within the lumbar spinal cord.

The major discovery of this first task will be identifying which pathway and/or pathways contribute to spontaneous recovery after injury. It will mostlikely be due to surviving axons from several pathways. To determine which ones contribute to recovery we have begun injections of HiRet/lentivirus expressing an inducible form of tetanus toxin to silence individual pathways we have labeled using HiRet/GFP/lentivirus. We were hoping to start these studies early, mid-July, but the behavioral analyses on the control contusion animals took longer than expected, mostly because of technicians and students vacations. We recently started injecting the first cohort of these animals and will be evaluating them within the next few months.

4. Impact:

The brainstem areas with the most HiRet-GFP expression in contused animals are the pontine and medullary reticular formation in which expression is relatively robust. The pontine reticulospinal tract is a ventromedial tract that has excitatory influence on the muscle for weight support. After the first week of injury moderately severe contused rats typically show a BBB score of about 6. The hind limbs at this score show no weight support but movement along 2 joints. After a 4 week recovery period, functional recovery improved and reached a maximum score of about 11. At this score the animal shows frequent stepping with weight support but little forelimb/hindlimb coordination, however, the rump of the animal is lower to the ground than normal. The ability to weight support is most likely partially mediated by the surviving axons of the pontine reticulospinal tract. To verify this we will silence these neurons and determine if animals lose their ability to weight support. We can then determine if increasing the plasticity of this pathway (Task 2) increases the weight support by measuring the height of the rump from the ground. We also observed about a 50% preservation of the medullary reticulospinal tract. This tract inhibits excitatory axial extensor muscles during movement. In general the reticulospinal tracts are involved in initiation of movement and their plasticity is important for locomotion. Lack of reinnervation of the vestibular nucleus is consistent with the animals lack of stability in both the BBB and ladder behaviors. Both normal and contused

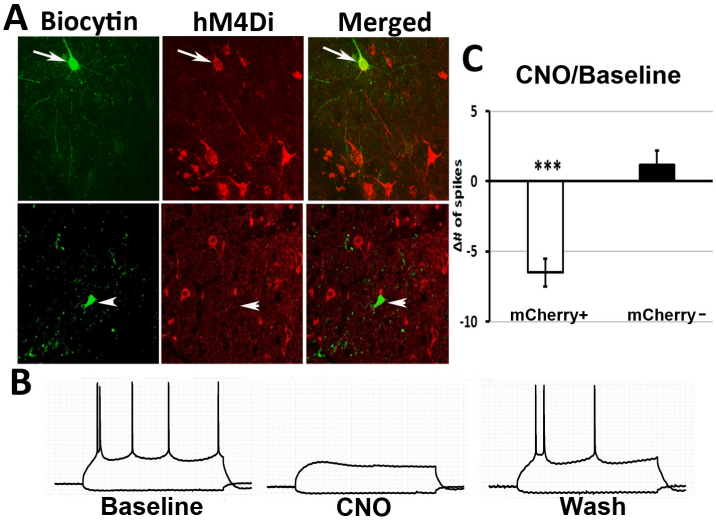
animals show labeling of a small but distinct population of Red nucleus neurons with HiRet-GFP. This nucleus may thus be important as an alternative pathway that connects with propriospinal neurons in the injured state. We observed highly levels of genetic tracer in propriospinal neurons in the cervical and thoracic region in normal animals. After contusive injury there was an 85% loss of thoracic propriospinal neurons and about a 50% loss of cervical propriospinal neurons. These neurons might be involved in interlimb coordination, which is still not well established in our animals. They might also function to support alternating stepping in the absence of ascending sensory information, which is lost after contusion injury. Silencing of this neuronal population will help to identify their potential role in mediating hindlimb patterning and as an alternative pathway mediating gait.

5. Changes/Problems:

Presently, we have only had a few problems associated with equipment and user time on the stereological system. Such problems do not diminish the experimental design, methods or potential outcomes, but might delay analysis, which is frustrating.

In addition to examining supraspinal sprouting into the cervical cord, we would also like to expand this aim to include examining sprouting of corticospinal axons into the pontine and medullary reticular formation. Such sprouting could increase the drive of these neurons to further promote recovery of function mediated by reticulospinal axons bypassing the lesion.

As mentioned in the previous report, we have generated AAV to selectively express inhibitor dreadds (designer receptors exclusively activated by designer drugs) to silence these neuronal pathways. The advantage of dreadds over inducible tetnus toxin is that the receptors can be constitutively expressed in neurons and selectively turned on or off using clozapine-N-oxide (CNO), which has no known effect of neuronal function in neurons not expressing inhibitor dreadds (hM4Di). We have tested our viruses both in vitro and in vivo and have observed excellent neuronal silencing (Figure 6). Figure 6 shows rat cortical slices expressing hM4Di/mCherry (A, red) and patch clamped neurons backfilled with biocytin (green). Spontaneous neuronal firing of hM4Di expressing neurons were silenced with addition of CNO (B). Only neurons expressing hM4Di were silenced by CNO (C). This method will be used either in conjunction with inducible-tetnus toxin or if the inducible-tetnus toxin fails to show behavioral loss when activated.



6. Products:

None at this time.

7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

None at this time