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13. SUPPLEMENTARY NOTES

14. ABSTRACT

This is the second 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 subtasks 1 for specific aim 2. This Aim 2 of the study, we proposed to examine regeneration and sprouting of supraspinal axons after treatment with our PTEN Antagonist Peptides (1-4). We have previously observed PAP2 to induce robust regeneration in a dorsal hemisection model and thought it might enhance regeneration and sprouting after the more clinically relevant severe contusion injury. Unfortunately, upon completion of the study we did not observe enhanced functional recovery between controls and PAP treated groups. We observed some differences in corticospinal axon tract regeneration and sprouting in the presence of PAP2 compared to PAP1 and PAP4, but not vehicle controls. In addition we observed no statistically significant differences in serotonergic sprouting between controls and PAP treated animals. We are presently starting specific aim 3 of the study to examine enhancing propriospinal axon sprouting using GDNF alone or in combination with NT-3.

15. SUBJECT TERMS

None listed

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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:

Major Task 1: All components of this task have been completed and we are presently finishing the manuscript for submission later this month.

Subtask 1.1: We received ACURO approval to begin animal studies.
Subtask 1.2: We have performed all the histology and we are finishing the stereology on these labeled neurons to determine if there are differences in their numbers between normal and

contused rats. The stereology should have been completed, but we ran into a software problem that took several weeks to solve. We have the majority of this data quantified and should be finished with this part by the end of this month. We are also correlating the changes in neurons bypassing the lesion with the hypothesized behavioral contribution of that pathway. So far, the retrograde labeling correlates well with the observed behavior.
Subtask 1.3: We observed highly levels of genetic retrograde tracer and quantified the changes in neurons before and after injury within multiple locations such as propriospinal neurons; reticulospinal neurons, rubrospinal neurons. We have observed about 30 – 50% of the neurons surviving within these particular regions. This data is published in (Sheikh et al., 2018; Keefe et al., in press)
Subtask 1.4: We have performed anterograde tracing and postsynaptic neurons expression studies using AAV coexpressing mCherry and WGA. Although the background was initial high we have improved staining and identified WGA transfer to some propriospinal neurons in the thoracic cord.
Subtask 1.5: We have completed the behavioral studies after neuronal silencing, however the data is presently inconclusive to which surviving neuronal pathway is mediating recovery. It appears that silencing each pathway reduced behavior a small amount but none showed a statistically significant decrease. This may be due to the fact that recovery requires multiple pathways each contributing to locomotion. We will attempt to silence multiple pathways and examine several other behavioral measures to analyze finer details of function for each of the pathways bypassing the lesion. Some of this data is also published in Sheikh et al., (2018).

Major Task 2: Examine the contribution of generalized supraspinal sprouting for connectivity onto propriospinal neurons to promote functional recovery.

Subtask 2.1: We have completed the study to examine the functional contribution of PTEN antagonist-induced supraspinal sprouting for promoting hindlimb locomotion. Four PTEN antagonist peptides (PAPs 1-4) targeted at different amino acid sequences of the PTEN protein were infused into our animals beginning 5 days post injury. We found robust axonal growth and sprouting caudal to a contusion in a subset of animals infused with PAPs 2 and 3, including typical morphology of growing axons. Serotonergic fiber growth was unaffected by peptide infusion and did not correlate with CST fiber density. Though some variability was seen in amount of growth within our animal groups, we find these PTEN antagonist peptides to be a promising and clinically relevant tool to encourage CST sprouting, and may be a useful addition to combination therapies.
Subtask 2.2: Quantify the extent to which increased supraspinal sprouting induces more synaptic connections onto propriospinal neurons bypassing the lesion. Examination of CST sprouting onto propriospinal neurons did not show statistically significant increases. This was mostlikely related to the extensive cell death of thoracic propriospinal neurons observed after contusion in these animals.
This aim is complete and the study is in preparation for publication.

Major Task 3: Examine whether sprouting of propriospinal neurons will promote functional recovery of hindlimb locomotion.

For Task 3.1, we have begun studies to determine if overexpression of GDNF can induce sprouting of cervical propriospinal axons caudal to a T10 moderate contusion injury. GDNF is a well-established neurotrophin that we have previously shown to induce robust sprouting and regeneration of propriospinal neurons through conduits of Schwann cells implanted into the contusion site of the lesioned spinal cord

(Deng et al., 2013; J. Neurosci., 33:5655). In that study although we observed very good growth, functional recovery was limited because the axons preferred to remain within the Schwann cell graft and only a few regrew back into the spinal cord. In this study, we will determine if expression of GDNF caudal to the lesion will induce sprouting of the surviving propriospinal neurons within the cord to promote functional recovery. The reason we chose to examine cervical propriospinal neurons is that in a recent published paper supported by this grant, we observed extensive numbers of cervical propriospinal neurons bypass the lesion site (Sheikh et al., 2018 Front Neural Circuits, Jul 25;12:60). The first cohort of rats for task 3 has been completed and data is being analyzed. For this experiment, AAV/GDNF or AAV/mCherry was injected into the L1 – L3 region of the spinal cord one week prior to T10 spinal contusion injury. This was a moderate, 200 kilodyne spinal contusion injury created using the infinite horizon device. Six weeks later, we injected the high efficiency retrograde lentivirus tracer HiRet/GFP into lamina VII, VII and IX of the lumbar spinal cord (L1 –L3). Figure 1, shows the results from 4 representative rat spinal cord. Cervical spinal cord of rats injected with the control AAV (control A & B) showed fewer GFP labelled propriospinal neurons than observed when compared to injections of adeno-associated virus encoding GDNF. The GDNF injected rats showed many more labeled cervical propriospinal neurons, indicating GDNF either increase the sprouting of propriospinal axons around the lesion or increase the survival of propriospinal neurons. This data is being quantified to determine if the increased numbers of labeled neurons bypassing the lesion between the GDNF and control groups are statistically significant. We have begun injecting our second cohort of GDNF rats. These rats will receive treadmill training and the level of functional recovery determined.

Figure 1, GDNF enhanced PNs labeling

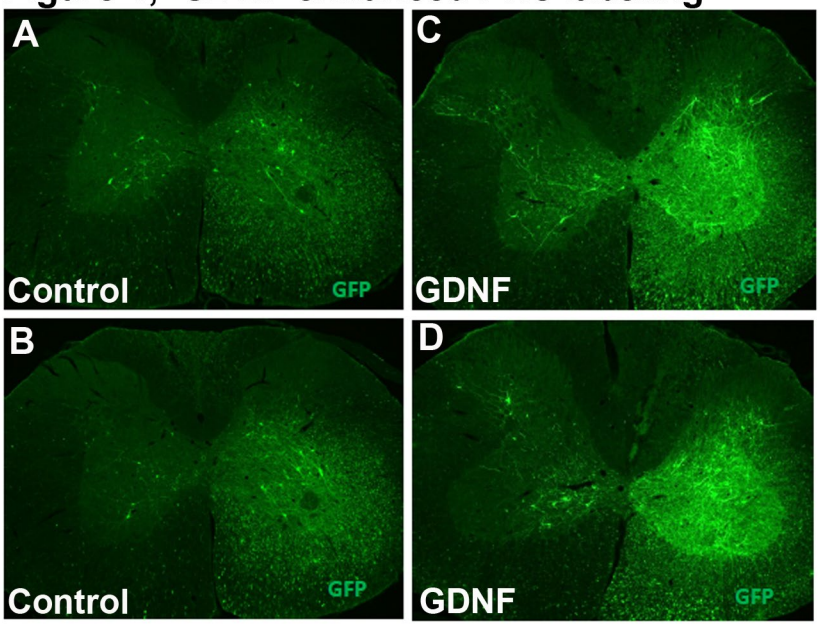
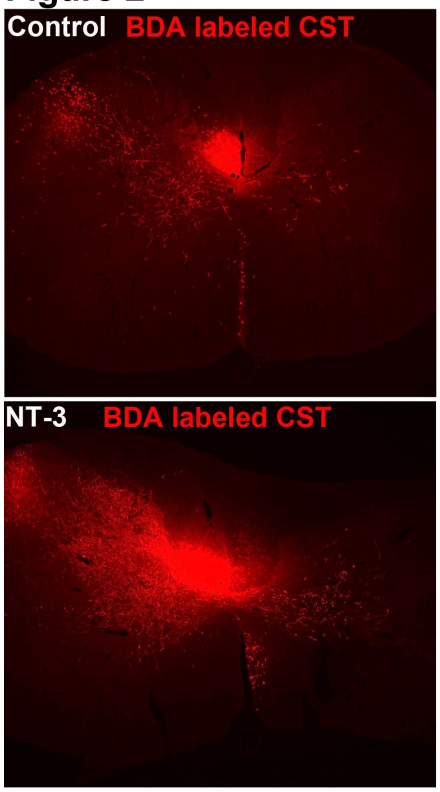
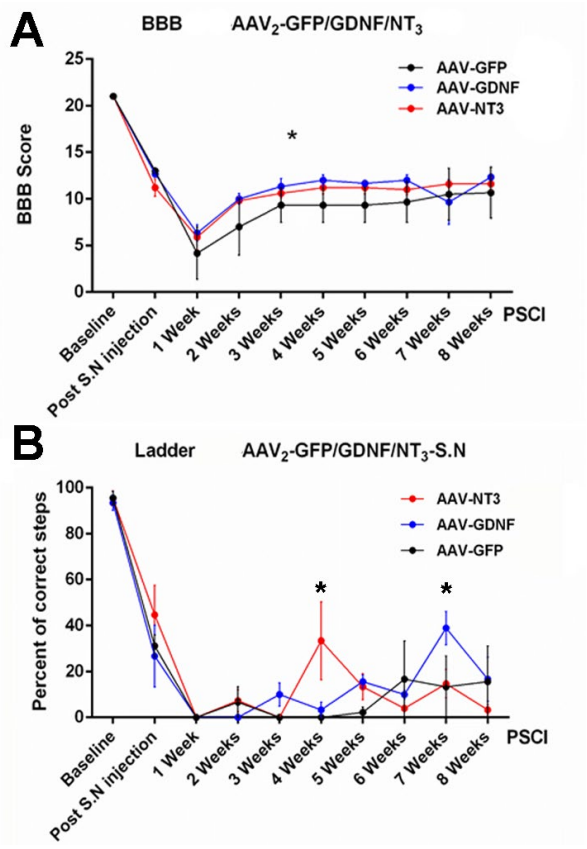


Figure 2



The second part of this task (3.2) has been approved to include NT-3 expression. This is to enhance the effectiveness of the propriospinal circuit bypass. Expression of NT-3 by cervical propriospinal neurons should attract lesioned corticospinal tract axon to sprouting onto them. The overall goal is to create a relay circuit bypassing the lesion that relays corticospinal tract commands past the lesion site via propriospinal neurons surviving the injury. Here GDNF will be expressed as in task 3.1 with the addition of expressing NT-3 by propriospinal neurons in the cervical spinal cord to attract

corticospinal tract axons to make synaptic connections. Figure 2 shows AAV/NT-3 induced sprouting of CST axons pre-labeled by the anterograde axon tracer BDA. The left images shows BDA labeling of axons terminals extending into the control cervical spinal cord, where they synapses onto many local interneurons and propriospinal neurons. The image to the right shows extensive sprouting of CST axons within the cervical spinal cord. These axons were observed to sprout both ipsilaterally and contralaterally to the expression of NT-3 (AAV/NT3), which was injected into the left side of the spinal cord.



The last component of this aim was to examine recovery of locomotor function after treatment with GDNF, NT-3 or GDNF + NT3 as described above and moderate contusive injury to the T10 spinal cord. In this study, GDNF showed statically significant increased functional return in the BBB, except at 7 weeks, when compared to GFP controls, but not after NT-3 treatment (Fig 3). Groups treated with GDNF and NT-3 were very similar to GDNF alone (not shown), however, by histological analyses we observed very low NT-3 staining within the spinal cord, indicating poor NT-3 expression. We also observed some minor improvement in hindlimb placement of rungs of the ladder grid walkway. Although, we are at the end of this project, we still will complete the GDNF/NT-3 study since we think it may still show beneficial outcomes above that of GDNF alone.

4. Impact:

For the beginning of the studies for Task 3, we showed very good preservation of propriospinal neurons bypassing the contusion lesion, which appears enhanced by the expression of GDNF. We will use our HiRet lentiviral vector to induce selective expression of NT3 from these propriospinal neurons bypassing the lesion to attract CST sprouting directly onto them. We were expecting expression of NT-3 in combination with GDNF caudal to the lesion to enhance relay formation form sprouting of CST axons onto cervical propriospinal neurons or the sprouting of propriospinal axons distal to the lesion. We tested both of these viruses and observed increase sprouting of both populations in accordance with the neurotrophin and placement within the spinal cord. Both GDNF and NT-3 alone showed increased recovery by the BBB, however, only the recovery mediated by GDNF was statistically significant. Although we have observed the expression of NT-3 by propriospinal neurons in the cervical region, when combined with GDNF, it was very poor and was most likely expressed at a non-functional level. This was most likely due to injection errors that were not identified until final analyses. Eventhough this project is complete, we plan on repeating the GDNF/NT-3 injections, since we still think the combination should support better functional recovery than either alone.

5. Changes/Problems:

Project finished

6. Products:

Sheikh IS, Keefe KM, Sterling NA, Junker IP, Eneanya CI, Liu Y, Tang QX, Smith GM. Retrograde transportable lentivirus tracers for mapping spinal cord locomotor circuits. Frontiers in Neural Circuits. 2018 Jul 25;12:60.

Keefe KM, Junker IP, Sheikh IS, Smith G M. Direct Injection of Highly Efficient Retrogradely Transportable Lentivirus Highlights Multiple Motor Pathways in the Rat Spinal Cord. JOVE, In press.

Kathleen KM, Junker IP, Eaenya CI, Tan Z, Berke J, Li S, Smith GM. The Effects of PTEN Antagonist Peptides on Growth of Corticospinal Axons in a Contusion Model of Spinal Cord Injury. Submitted