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TITLE:  Spinal Cord Injury-Induced Dysautonomia via Plasticity in Paravertebral Sympathetic Postganglionic

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ABSTRACT  Sympathetic postganglionic neurons (SPNs) located in sympathetic ganglia represent the final common sympathetic motor output. Even though SCI produces a profound plasticity in sympathetic autonomic function, the extent that SCI-induced dysautonomia is based on SPN changes within the thoracic paravertebral sympathetic chain is unknown. Given their strategic site in autonomic signaling to body, any plasticity is likely to be of high significance, yet there is a paucity of studies undoubtedly due to their near anatomical inaccessibility. We have solved the accessibility problem with a strategic methodological advance. We will determine the extent to which paravertebral SPNs are a nodal site for vasomotor dysfunction after SCI. We will undertake physiological, pharmacological and optogenetic studies to examine network and cellular plasticity induced by SCI to answer the following two questions: (a) Does SCI lead to plasticity in synaptic interactions between preganglioniccs, SPNs and primary afferents? (b) Do SPNs become hyperresponsive to synaptic inputs after SCI?
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1. **INTRODUCTION:**

   Sympathetic postganglionic neurons (SPNs) located in sympathetic ganglia represent the final common sympathetic motor output. Even though SCI produces a profound plasticity in sympathetic autonomic function, the extent that SCI-induced dysautonomia is based on SPN changes within the thoracic paravertebral sympathetic chain is unknown. Given their strategic site in autonomic signaling to body, any plasticity is likely to be of high significance, yet there is a paucity of studies undoubtedly due to their near anatomical inaccessibility. We have solved the accessibility problem with a strategic methodological advance. We will determine the extent to which paravertebral SPNs are a nodal site for vasomotor dysfunction after SCI.

   We will undertake physiological, pharmacological and optogenetic studies to examine network and cellular plasticity induced by SCI to answer the following two questions: (a) Does SCI lead to plasticity in synaptic interactions between preganglionics, SPNs and primary afferents? (b) Do SPNs become hyperresponsive to synaptic inputs after SCI?

2. **KEYWORDS:**

   spinal cord injury, sympathetic, autonomic, autonomic dysreflexia, spinal cord, electrophysiology, plasticity, paravertebral, postganglionic
3. ACCOMPLISHMENTS:

The PI is reminded that the recipient organization is required to obtain prior written approval from the awarding agency Grants Officer whenever there are significant changes in the project or its direction.

a. What were the major goals of the project?

1. List the major goals of the project as stated in the approved SOW. If the application listed milestones/target dates for important activities or phases of the project identify these dates and show actual completion dates or the percentage of completion.

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*Milestone(s) Achieved:* Understanding of synaptic organization in uninjured mice and ability to use optogenetics to selectively activate afferent and efferent fiber populations

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*Milestone(s) Achieved:* Demonstration of important contribution of thoracic sympathetic chain to SCI-induced autonomic plasticity and forward insight into therapeutic interventions for future study

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*Milestone(s) Achieved:* Dissemination of scientific results.
b. What was accomplished under these goals?

- major activities; 2) specific objectives; 3) significant results or key outcomes, including major findings, developments, or conclusions (both positive and negative)

Accomplishments under specific sections are described below followed by an overall annual summary that synthesizes these accomplishments. Please refer to figures in the overall summary as needed.

1a.1: Segment specific properties

Methods/experiment: Mice are euthanized (.2mL 50% urethane) and thoracolumbar spinal column quickly removed. The vertebral column is cut longitudinally, both dorsally and ventrally, and spinal roots are severed to remove spinal cord. Remaining vertebral column and ribs are trimmed to include only the thoracic region*. The tissue is pinned down in a sylgard recording chamber and suction electrodes are positioned to stimulate various thoracic ventral roots and record from various thoracic ganglia.

Progress/results: Extracellular recordings show that there is a convergence onto individual ganglia. For example, stimulating T4-T11 ventral roots results in activity in the T11 ganglion (Fig. 7A in overall summary below). If we repeat this with many ganglia, a more complete picture emerges, showing that there is also significant divergence of ventral root input to thoracic ganglia (Fig. 7C in overall summary below). We aim to repeat these trials using optical stimulation of ventral roots. We are currently training an undergraduate student to help with these experiments.

1a.2: Pharmacology

Methods/experiment: Dissected vertebral column described in the methods section above is pinned down in recording chamber with stimulating suction electrodes on various ventral roots and a recording electrode on thoracic ganglia. We have been testing for synaptic transmitter identity by applying glutamatergic, cholinergic, nitrergic, purinergic and adrenergic ionotropic receptor antagonists to the recording chamber.

Progress/results: Extracellular Recordings. We have found evidence for a contribution from glutamatergic, nitrergic and cholinergic transmission in both ventral root and dorsal root evoked responses. Postganglionic transmission is thought to occur via nicotinic acetylcholine receptor subunits. We have conducted experiments with nAChR antagonists that act on different receptor subunits and have found reduction from baseline synaptic transmission. Our next step is to increase the sample size.

Intracellular Recordings. Experiments are underway to analyze the effects of various channel blockers on intrinsic membrane currents in intracellular recordings from individual neurons.

1a.3: Breeding/crossing transgenic mice and spinalizations

Methods/experiment: Standard animal husbandry

Progress/results: We currently have a healthy colony of ChAT-IRES-Cre::ChR2 mice available for performing in vitro optogenetic studies. We believe these mice will be more suitable than the BAC transgenics we previously used due to the more precise nature of their transgene insertion. These mice are used for all studies, with the exception of subtask 2.2. Subtask 2.2 will require the generation of Advillin::ChR2 mice to study afferent-postganglionic interactions. We are in possession of the requisite mouse strains, but have refrained from crossing them until other subtasks have nearing completion.

Spinalizations are behind schedule. The difficulty of caring for injured mice compounded with the relatively low success rate of our intracellular recording technique has slowed progress in this area. However, we plan to begin spinalization surgeries in earnest in early 2017. We hired and trained a new technician to help with animal care and have optimized our recording technique to moderately improve success rate.

1a.4: Establish intracellular recording techniques

Methods/experiment: Starting with the preparation to isolate the thoracic chain and after ribs and vertebrae are trimmed (see 1a.1 methods, *) the entire tissue is incubated at 37°C in collagenase for 1.5 hours. The tissue is then washed in physiological saline. Sympathetic chain is removed by severing rami and transferred to a recording chamber. Chain is pinned down in Sylgard, connective tissue is removed by scraping lightly with an insect pin, and recorded using standard patch clamp technique.

Progress/results: We are currently able to achieve acceptable recordings from most mice used in experiments, with recordings lasting at least 5-10 minutes. This is sufficient time to characterize basic cellular properties (i.e. input resistance, cell capacitance, basic firing properties, etc.) However, longer recordings are required to characterize convergent synaptic input properties and to study membrane current pharmacology. To date, high fidelity, long lasting recordings are admittedly rarer. Much of this is due to connective tissue and glia that cover
neuronal surfaces and make stable seals difficult to achieve. To compensate, we have simply increased the number of mice from which we attempt to record. Progress overall has been steady, but still slower than we had hoped.

1b.1: Incorporation of optogenetic approaches for selective activation of neuron populations

Methods/experiment: We have developed a laser-diode based stimulator which allows for optical activation of preganglionic axons in ChAT::ChR2 mice. Light can be directed to illuminate ventral roots (primarily for extracellular recordings), interganglionic nerve, or thoracic ganglia.

Progress/results: Evoked synaptic response fatigues due to repeated stimulation, and takes seconds to recover (Fig. 6C in overall summary below). When the entire ganglion is illuminated, eliminating any significant propagation delay, an interesting relationship emerges between excitatory postsynaptic current (EPSC) amplitude and latency (time between optical stimulus and the start of an evoked EPSC). Shorter latency corresponds to greater amplitude (Fig. 6D in overall summary below). When the interganglionic is illuminated, we have shown that different preganglionics are activated at different thresholds (Fig. 6E in overall summary below). We have also shown that a single cell receives input from multiple spinal cord segments (Fig. 7D in overall summary below).

2.1: Physiological plasticity in preganglionic-postganglionic interactions assessed using optogenetics

Methods/experiment: Methods described in 1b.1 are repeated in spinal cord injured mice.

Progress/results: Progress has been slow in this area. Tissue from injured mice appears to be more difficult to patch, i.e. high resistance seals are hard to achieve and recordings are "leaky." In light of this observation, we intend to stain the tissue for extracellular matrix components (collagen, chondroitin sulfate proteoglycans) to test the hypothesis that the extracellular matrix becomes denser after SCI. As stated previously, we have hired a new technician to help streamline the injury and recording process.

2.2: Physiological plasticity in afferent-postganglionic interactions assessed using optogenetics

Methods/experiment: N/A

Progress/results: N/A

2.3: Physiological plasticity in preganglionic-afferent interactions assessed using optogenetics

Methods/experiment: N/A

Progress/results: N/A

2.4: Intracellular recordings of synaptic and cellular plasticity in membrane properties; demonstration of membrane bistability

Methods/experiment:

Progress/results: SCI may induce greater frequency of spontaneous synaptic events. However, we currently have n=2 so this must be replicated before we can say this with confidence.

3.1: Data analysis

Methods/experiment: Data is analyzed in Clampfit, MATLAB, and/or Excel.

Progress/results: Basic cellular properties (input resistance, membrane capacitance, time constant, firing rate) have been analyzed. Analysis of synaptic properties are in progress.

3.2: Manuscript writing and submission

Methods/experiment: N/A

Progress/results: Manuscript writing is in progress. The abstract and methods sections are essentially complete. The results section is still in progress.

Overall summary: Sympathetic preganglionic neurons (SPNs) represent the final common sympathetic motor output. Thoracic SPNs (tSPNs) located in paravertebral chain ganglia control vasomotor function in trunk and upper extremities (Fig 1A). Their plasticity is likely to be of high clinical significance. Yet tSPNs are inaccessible for in vivo study, so operational principles are inferred from studies in cervical and lumbar chain ganglia (Percy and Krier, 1987; Bratton et al., 2010; Campanucci et al., 2010; Rimmer and Horn, 2010; Springer et al., 2015). To date, there are only 3 in vitro studies on tSPN physiological properties (Blackman and Purves, 1969; Lichtman et al., 1980; Jobling and Gibbins, 1999), and no accurate recordings of their cellular...
integrative properties or underlying recruitment principles. We developing an ex vivo adult mouse preparation with intact segmental preganglionic and rostrocaudal interganglionic connections and obtained the FIRST WHOLE CELL RECORDINGS of tSPN synaptic and cellular properties. Observed synaptic integrative and firing properties were fundamentally different than previously observed with sharp electrodes due to impalement injury (Jobling and Gibbins, 1999; Springer et al., 2015), and finally provide the critical prerequisite to understand tSPN cellular integrative and recruitment principles as a launchpad to interrogate mechanisms that generate abnormal increases in excitability including after spinal cord injury (SCI).

Most thoracic paravertebral sympathetic postganglionic neurons (tSPNs) control vasomotor function in upper and middle extremities of the trunk. This includes vascular supply to integumentary, cardiorespiratory and digestive systems. Whereas prevertebral sympathetic ganglia are typically associated with one or more visceral organs in a discrete location, chain ganglia can be thought of as a distribution system for sympathetic activity that must span the body vasculature.

One important issue is whether sympathetic postganglionic neurons (SPNs) are driven directly from preganglionic neurons giving a simple chain of command. Alternatively, postganglionic neurons can act as integrators, making the information processing system more complex during recruitment. A summary of their anatomical organization is shown (Fig 2). They are presumably excited by sympathetic preganglionic neurons via the ‘n+1’ rule. The ‘n+1’ rule states that the postganglionic neurons receive one (1) primary synapse from a preganglionic neuron, and several (‘n’) secondary synapses which are smaller and have little effect on postganglionic firing properties. This organizational principle of recruitment is believed to dictate recruitment of postganglionics in superior cervical (Rimmer and Horn, 2010) and lumbar sympathetic chain ganglia (McLachlan, 2003). Consequently, SPNs have traditionally been considered to behave as 1:1 relays of preganglionic commands.

UPDATE. (A) Characterization of cellular properties in adult mouse thoracic paravertebral ganglia. [Fig 3 & 4] A major function of sympathetic paravertebral chain ganglia neurons is to maintain vasomotor tone. While the functional properties of cervical and lumbosacral paravertebral ganglia neurons have been characterized, little is known about the functional properties of neurons within thoracic paravertebral ganglia. We developed an approach that allows for whole-cell patch clamp recordings in intact thoracic ganglia to characterize cellular and synaptic
We recorded from 12 cells deemed of good quality and obtained the following mean values ± SD: resting membrane potential (-57 ± 9 mV) [Fig. 4A], membrane resistance (985 ± 501 MΩ), and τ_m (99 ± 49 ms) [Fig. 4B]. Threshold voltage was typically 10 mV higher than resting membrane potential, action potentials displayed after-hyperpolarization and some cells displayed post-inhibitory rebound. All neurons were capable of repetitive firing. Maximal firing rates observed in response to depolarizing current steps ranged from 14-17 spikes/sec. During intracellular depolarization, firing rate increases with increased current injection and cells sustain tonic firing. Spike frequency adaptation was also observed. All recorded properties are fully consistent with those reported recently with whole cell recordings in rat superior cervical ganglia (Springer et al., 2015). Strikingly, our recorded properties differ substantially from sharp electrode recordings obtained from adult mouse tSPNs (Jobling and Gibbins, 1999). Our recorded membrane resistance is 4.5 fold higher and τ_m is 7.5 fold longer than observed by Jobling and Gibbins (1999), and our neurons only fired tonically (e.g. Fig 4) while theirs only fired phasically to depolarizing current pulses. Note that all of these differences are consistent with greater cell damage caused by sharp electrode penetration compared to patch clamp recordings. We therefore assume that our new whole cell recordings are closer to physiological reality.

(B) Synaptic and anatomical properties of thoracic sympathetic chain ganglia.

Synaptic properties of paravertebral neurons. Spontaneous EPSP amplitudes occupy a continuous range. For the neuron shown, this was from 1-8mV (Fig 5A,B). Previous studies implied that due to the short time constant of postganglionic neurons, synaptic integration has a negligible effect on cell firing. Using patch clamp recordings, the measured time constant of cells is longer, so we observe that spontaneous coincident EPSPs often sum and lead to an action potential (Fig 5C).

Using (ChAT)::channel rhodopsin (ChR2) mice, we optogenetically recruited either cholinergic preganglionic axons in the interganglionic nerve or preganglionic synapses within thoracic ganglia (Fig 6). Activation of preganglions by blue light illumination leads to a massive EPSC (~500pA) that fatigues rapidly with repetitive 10Hz stimulation (Fig 6B). As stimulation frequency increases, synaptic currents show considerable fatigue. This may indicate that presynaptic release of acetylcholine attenuates rapidly (on the order of 10-300ms) and this is the rate-limiting step for transmission in tSPNs. Rate of recovery after fatigue was also characterized using a paired pulse protocol (Fig 6C). The effects of synaptic fatigue are apparent even after 5 seconds of rest. We also obtained preliminary evidence for the existence of a size principle of recruitment. We observed that spontaneous coincident EPSPs often sum and lead to an action potential (Fig 5C).

Properties of preganglionic divergence and convergence onto thoracic chain SPNs was previously examined in guinea pig (Blackman and Purves, 1969; Lichtman et al., 1980). Spinal cord preganglionic neurons project axon collaterals intersegmentally for divergent actions on many tSPNs. Conversely, tSPNs within an individual ganglion...
receive preganglionic convergent input from multiple spinal segments (Blackman and Purves, 1969; Lichtman et al., 1980). In the superior cervical ganglion, individual SPNs receive input on average from ~9 preganglionic neurons (Purves and Lichtman, 1985). In comparison, the firing of postganglionic in rodent lumbar ganglia may be ‘normally’ driven by 2 to 3 preganglionic neurons (Bratton et al., 2010). We began to undertake similar studies in the adult mouse and observed comparable patterns of convergence and divergence. Recruited of presynaptic events covered a comparable range of conduction velocities (Blackman and Purves, 1969)(Fig 7A-C). We have also begun to assess convergence properties onto individual tSPNs (Fig 7D).


4) other achievements.

Difficulty in obtaining recordings from spinal cord injured tissue.

We’ve had considerable difficulty in obtaining access to the cellular properties of these neurons after spinal cord injury. One possibility is that the injury leads to the generation of novel structural/cellular components that surround sympathetic ganglia. The working hard at trying to modify experimental approach and have begun to obtain success in the last month. This data has yet to be analyzed. Having said that recording quality has still been suboptimal and we have just ordered dispase as an additional protease to apply in conjunction with collagenase in an attempt to make the neuronal tissue more accessible.

c. What opportunities for training and professional development has the project provided?

  o One individual was sent to a specialty meeting on spinal cord function in Marseille France to present his work and two individuals are being sent to the Annual Society for Neuroscience Meeting in San Diego this November.

d. Describe briefly what you plan to do during the next reporting period to accomplish the goals and objectives.

  o Continue investigations as proposed with much more focused effort on anatomical and cellular plasticity after spinal cord injury.
  o We are now accumulated several ganglia for anatomical assessment but have yet to begun anatomical analysis.

4. IMPACT:

  o What was the impact on the development of the principal discipline(s) of the project?
    ▪ Nothing to Report
  o What was the impact on other disciplines?
    ▪ Led to a CRCNS application with a computational neuroscientist.
    ▪ Led to a R01 application with a computational neuroscientist
  o What was the impact on technology transfer?
    ▪ Nothing to Report
  o What was the impact on society beyond science and technology?
    ▪ Nothing to Report.

5. CHANGES/PROBLEMS:

Nothing to Report

6. PRODUCTS:

Nothing to Report

Publications, conference papers, and presentations

Other publications, conference papers, and presentations. Identify any other publications, conference papers and/or presentations not reported above. Specify the
status of the publication as noted above. List presentations made during the last year (international, national, local societies, military meetings, etc.). Use an asterisk (*) if presentation produced a manuscript.


7. PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS

What individuals have worked on the project?

- Mallika Halder – 25% effort – research specialist
- Michal McKinnon – 90% effort – graduate student
- Michael Sawchuk, - 50% effort - lab manager

e. Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

- PI. Craig H Neilsen Foundation. Continuous sensor-based home-cage recordings for SCI research. 10/16-10/19, $600,000 total.
- Co-PI. [Garraway PI] Craig H Neilsen Foundation. Compromised Aδ-LTMRs function contributes to alldynia after SCI 8/16-8/18, $300,000 total.

f. What other organizations were involved as partners?

- Nothing to Report

8. SPECIAL REPORTING REQUIREMENTS
9. APPENDICES:

g. abstracts,
PATCH CLAMP RECORDINGS OF CELLULAR AND SYNAPTIC PROPERTIES IN ADULT MOUSE THORACIC PARAVERTEBRAL GANGLIA

M.L. McKinnon, M. Halder, S. Hochman
Dept. Physiol, Emory Univ Sch Med, Decatur, GA

BACKGROUND

1. Paravertebral sympathetic ganglia are essential for normal body homeostasis and have been implicated in the onset and progression of various diseases.
2. Recent studies have highlighted the importance of understanding the cellular and synaptic properties of these ganglia.

ANATOMICAL SCHEMATIC

A schematic representation of the sympathetic chain is shown, with emphasis on the thoracic paravertebral ganglia.

POSTGANGLIONIC PROPERTIES

Passive membrane properties

A. Patch clamp recordings reveal passive membrane properties of paraganglion neurons. B. Comparison of input resistance, resting membrane potential, and membrane time constant across different sympathetic ganglion segments. C. Plot showing the relationship between input resistance and resting membrane potential.

Active membrane properties

A. Patch clamp recordings reveal active membrane properties of paraganglion neurons. B. Comparison of action potential amplitude and duration across different sympathetic ganglion segments. C. Plot showing the relationship between action potential amplitude and duration.

SYNAPTIC ACTIVITY

Spontaneous synaptic activity

A. Oscilloscope traces of spontaneous synaptic activity. B. Comparison of spontaneous synaptic activity across different sympathetic ganglion segments. C. Plot showing the relationship between spontaneous synaptic activity and sympathetic ganglion segment.

Evoked synaptic activity

A. Oscilloscope traces of evoked synaptic activity. B. Comparison of evoked synaptic activity across different sympathetic ganglion segments. C. Plot showing the relationship between evoked synaptic activity and sympathetic ganglion segment.

DISCUSSION

1. Sympathetic nerve activity is regulated by neural and humoral factors, which may affect the synaptic transmission in these ganglia.
2. Recent studies suggest that alterations in synaptic transmission may contribute to the pathogenesis of various diseases.

REFERENCES