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NEURONAL PROGENITOR TRANSPLANTATION AND RESPIRATORY OUTCOMES FOLLOWING UPPER CERVICAL SPINAL CORD INJURY IN ADULT RATS

Todd E. White^{1,*}, Michael A. Lane¹, Milapjit S. Sandhu², Barbara E. O'Steen¹, David D. Fuller², and Paul J. Reier^{1,**}

¹University of Florida College of Medicine and McKnight Brain Institute, Department of Neuroscience, P.O. Box 100244, 100 S. Newell Dr., Gainesville, FL. 32610, U.S.A.

²University of Florida College of Health and Health Professions, Department of Physical Therapy, P.O. Box 100154, 100 S. Newell Dr., Gainesville, Florida 32610, U.S.A.

Abstract

Despite extensive gray matter loss following spinal cord injury (SCI), little attention has been given to neuronal replacement strategies and their effects on specific functional circuits in the injured spinal cord. In the present study, we assessed breathing behavior and phrenic nerve electrophysiological activity following transplantation of microdissected dorsal or ventral pieces of rat fetal spinal cord tissue (FSC_D or FSC_V, respectively) into acute, cervical (C2) spinal hemisections. Transneuronal tracing demonstrated connectivity between donor neurons from both sources and the host phrenic circuitry. Phrenic nerve recordings revealed differential effects of dorsally- vs. ventrally-derived neural progenitors on ipsilateral phrenic nerve recovery and activity. These initial results suggest that local gray matter repair can influence motoneuron function in targeted circuits following spinal cord injury and that outcomes will be dependent on the properties and phenotypic fates of the donor cells employed.

Keywords

rats; fetal spinal cord; ventilation; phrenic; transplantation; respiratory; cervical spinal cord injury

Introduction

Various cellular therapies are considered to have significant potential for promoting functional repair of the injured spinal cord (Eftekharpour et al., 2008, Reier, 2004). This is especially reflected by interest in transplantation of myelin-competent cells for remyelination of spared axons (Cao et al., 2010, Karimi-Abdolrezaee et al., 2010, Kocsis et al., 2004, Sasaki et al., 2007, Sharp et al., 2010). Less frequently considered, however, is the fact that focal neuronal loss after trauma or ischemic insult can also have functional

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****Corresponding Author Forward All Correspondence To:** Paul J. Reier, Ph.D. University of Florida College of Medicine Department of Neuroscience P.O. Box 100244 Gainesville, FL. 3210 reier@mbi.ufl.edu Phone: 352-392-8696.

***Current Address:** Neuroscience Institute, Morehouse School of Medicine, 720 Westview Drive, SW., Atlanta, GA 30310, U.S.A.

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consequences even when gray matter damage does not involve the spinal enlargements (Vierck et al., 2000, Yezierski et al., 1998) or when motoneuron pools are spared (Cizkova et al., 2007, Hadi et al., 2000, Magnuson et al., 2005).

Several laboratories have reported post-SCI functional improvements associated with intraspinal grafts containing neuronal progenitors either alone or in combination with other cells or interventions (Bonner et al., 2010, Hooshmand et al., 2009, Kim et al., 2006, Mitsui et al., 2005, Nikulina et al., 2004). However, little attention has been given to demonstrations of graft integration with functionally and neuroanatomically-defined spinal circuits and whether distinct donor neuronal phenotypes differentially affect recovery (Goldman and Windrem, 2006).

In the present study, neural tissue grafts, which were predominantly comprised of interneuronal precursors, were introduced into an acute, cervical (C2) spinal hemisection (Hx) model of respiratory compromise (Fuller et al., 2008, Goshgarian, 2003, Lane et al., 2008a). Recent pharmacological and neuroanatomical studies have led to the suggestion that interneurons in dorsal versus intermediate or ventral gray matter at the level of the phrenic nucleus may play contrasting modulatory roles in spontaneous recovery of ipsilateral phrenic motoneuron (PhMN) function following C2Hx injuries (Lane et al., 2009, Zimmer and Goshgarian, 2007). Therefore, we investigated whether grafts of dorsal or ventral regions of rat fetal spinal cord (FSC) tissue would differentially influence respiratory recovery post-C2Hx and whether connectivity between donor neurons and the host phrenic circuit could be demonstrated.

Methods

All surgical and animal care procedures were approved by the Institutional Animal Care and Use Committee at the University of Florida. Adult, female Sprague-Dawley, (205–265 g) rats were deeply anesthetized with xylazine (3 mg/kg; Phoenix Pharmaceutical, Inc., St. Joseph, MO) and ketamine (90 mg/kg; Fort Dodge Animal Health, Fort Dodge, IA). After laminectomy, a dural incision was made and hemisection cavity created at the C2 level (Jakeman and Reier, 1991). Post-hoc histological analysis confirmed the extent of hemisection and animals with spared ipsilateral tissue were excluded from the study (Fuller et al., 2009).

Pregnant Sprague-Dawley rats were anesthetized with xylazine and ketamine at 14 days of gestation (E13.5 – E14). Embryos were removed and meninges/dorsal root ganglion-free spinal cords were isolated in Hank's Balanced Salt Solution (Gibco) (Jakeman and Reier, 1991). Dorsal and ventral strips of solid FSC tissue were then microdissected from the extreme alar and basal plate regions, respectively, as shown in Figure 1A. Multiple FSC strips of dorsal (FSC_D; dorsal (alar plate) grafts, n=17) or ventral (FSC_V; ventral (floor-plate) grafts, n=15) tissue were then introduced into acute lesion cavities, followed by dural suturing and incision site closure (Jakeman and Reier, 1991). In addition, a group of C2Hx-only controls (n=9) were included in our respiratory analyses. Post-operative care included Lactated Ringers (5ml s.q.), yohimbine (0.4mg s.q.), and Buprenorphine (0.012mg/kg s.q.) for analgesia.

Whole body plethysmography was used to obtain repeated measures of breathing in unanesthetized rats (Buxco Inc., Wilmington, NC, USA) (for details, see Fuller et al., 2008), pre-injury, 2 and 12 weeks post-injury. Breathing frequency (F; breaths/minute), tidal volume (TV; ml/breath) and minute ventilation ($\dot{V}_E$; ml/minute) were calculated from the airflow traces. Baseline recordings were made for 60–90 min while the chamber was flushed with 21% O₂ (balance N₂). Rats were then given a 10 min hypercapnic challenge (7% CO₂,

21% O₂, balance N₂). Mean values were obtained from a 10 min period during baseline and during the last 5 min of the hypercapnic challenge (Fuller et al., 2006, Teng et al., 1999). Respiratory volume data (TV, $\dot{V}_E$) were normalized to body mass (Olson et al., 2001).

Efferent phrenic nerve compound action potentials were measured under controlled conditions (see Fuller et al., 2008). At the end of each experiment, rats were intracardially perfused with heparinized saline followed by 4% paraformaldehyde. Neurophysiology data were analyzed using Spike2 software (Cambridge Electronic Design Limited, Cambridge, England) as previously described (Fuller et al., 2009, Fuller et al., 2008).

Donor neuronal integration with the host phrenic circuitry (Lane et al. 2008) was examined in a subset of graft recipients (FSC_D, n=3; or FSC_V, n=3) using pseudorabies virus (PRV; Bartha strain; $\sim 8.0 \times 10^8$ pfu/ml) transneuronal tracing. Four days (96 hours) prior to euthanasia at 12 weeks post-injury, an incision was made at the level of the trachea, and the muscle overlying the brachial plexus ipsilateral to injury (and transplant) was deflected to expose the phrenic nerve. The intact nerve was then injected with PRV (~ 6 – 8μ l) using a Hamilton syringe (30g needle).

Spinal cord specimens were collected at 2–3 months post-transplantation to confirm the extent of lesion and for histological/immunocytochemical characterization of the transplants. Rats were euthanized with Beuthanasia solution (78 mg pentobarbital sodium and 10 mg phenytoin sodium) and perfused with either 4% paraformaldehyde or 3.5% glutaraldehyde / 4% paraformaldehyde in 0.1M phosphate buffered saline, for immunohistochemistry or histology in semi-thin sections, respectively. Cervical spinal cord tissue for immunocytochemistry was dissected and either cryoprotected for frozen sectioning or processed for vibratome sectioning. Tissue from animals used for PRV tracing were processed for vibratome sectioning and immunocytochemistry as described in detail in Lane et al. (2008b). Tissue from animals perfused with glutaraldehyde / paraformaldehyde combination was dissected and processed for plastic embedding (Lane et al., 2008b).

Immunostaining to demonstrate the distribution and packing density of neuronal nuclei (NeuN; Chemicon, 1:750) was performed to differentiate between dorsally vs. ventrally-enriched FSC grafts based on the appearance of superficial dorsal horn features of FSC transplants. These regions were previously shown to have characteristics of the substantia gelatinosa (SG) in terms of neuronal cytology and size, neuropeptide staining, and paucity of myelin (Jakeman et al., 1989). Visualization for immunohistochemistry was achieved with a VECTASTAIN® Elite ABC kit followed by reaction with diaminobenzidine tetrahydrochloride. All sections were examined and photographed with Zeiss Axioplan/ Axiophot microscopes.

Results

FSC_D transplants were characterized by numerous myelin-free areas (Fig. 1C, FSC_D), consisting of tightly-packed neurons (7–15 μ m in diameter), that were previously reported to have properties analogous to the substantia gelatinosa (SG) of the intact spinal cord (Jakeman et al., 1989). By comparison, ventrally-biased grafts were also more heavily myelinated, and neurons tended to be aggregated as pools of larger neurons separated by distinct fascicles of white matter coursing throughout these transplants (Fig. 1C, FSC_V).

To gain an impression of dorsal vs. ventral enrichment of grafted tissue the proportion of transplant area represented by “SG-like” regions was measured in randomly selected plastic sections taken from a subset of animals (FSC_D, n=4; FSC_V, n=5). Computer-assisted measurements were obtained with a KS400 Image Analysis System (Zeiss). These

measurements confirmed that FSC_D contained significantly more SG-like regions than FSC_V grafts (approximately 9% vs. 2%, respectively, $P < 0.001$; Fig. 1C, graph). The overall cytoarchitectural differences between the two donor tissues could also be visualized in tissue sections stained with NeuN (Fig. 1D). Multiple regions of clustered nuclei were found in FSC_D transplants, whereas far fewer were visible in the FSC_V counterparts. Unstained white matter fascicles separating neuronal aggregates were also more apparent in NeuN-stained sections of FSC_V recipients.

Measurements of cell diameters obtained from randomly selected plastic sections of graft tissue revealed the majority of neurons in FSC_V transplants were 15–30 μm in diameter, which approximated the distribution of interneuronal sizes sampled in specimens of normal adult intermediate and ventral gray matter (data not shown). A similar range applied to neurons outside of the SG-like regions in FSC_D transplants. Both dorsal and ventral E14 FSC grafts rarely contained cells resembling motoneurons in size (i.e., diameter $\geq 40 \mu\text{m}$) and shape (Sieradzan and Vrbova, 1989). Based on these collective observations, microdissected grafts prepared for this study appeared to be primarily enriched in ventral or dorsal interneuronal populations.

All animals included in the study exhibited grafts which substantially filled the lesion cavities with some degree of host-donor tissue approximation (Fig. 1D). Tissue confluence, however, occurred most consistently along donor tissue margins fused with host gray matter (near the central canal (arrows in Fig. 1D) and at the rostral-caudal borders of the transplant (dotted line in Fig. 1E)). Transneuronal labeling with PRV was used to test for host-graft neuronal integration. Injection of the virus into the phrenic nerve ipsilateral to lesion/transplant resulted in distinct labeling of the corresponding PhMN pool and bilateral distributions of spinal interneurons similar to what has been observed previously after topical PRV delivery to the ipsilateral hemidiaphragm (Lane et al., 2008b). Neuronal infection was also observed in both FSC_D and FSC_V transplants most commonly near the host-graft interfaces (Figs. 1E). This result corroborates previous results obtained with anterograde tracing methods (Jakeman and Reier, 1991) and further indicates that some donor neurons established synaptic connections with the phrenic circuit.

Plethysmography provides a measure of a subject's overall breathing pattern and $\dot{V}_E$ (see Lane et al., 2008a). Analysis of plethysmographic airflow traces from unanesthetized FSC_D and FSC_V rats (e.g. Fig. 2A) revealed no change in the rapid-shallow breathing pattern characteristically induced by C2Hx (Fuller et al., 2008, Fuller et al., 2006). Given the potential negative effect grafting could have at C2, it was noteworthy that no adverse impact was observed on the graft recipients' F (Fig. 2B), TV (Fig. 2C), or $\dot{V}_E$ (Fig. 2D) during eupneic baseline conditions (21% O₂, balance N₂). Statistical analyses showed no graft effect (all $P > 0.6$) when compared to lesion-only animals (2-way repeated measures analysis of variance (RM ANOVA); factor 1: time post-injury; factor 2: treatment [i.e. FSC_V, FSC_D, C2Hx]). Similarly, neither FSC graft affected F, TV (not shown) or $\dot{V}_E$ (Fig. 2E) during hypercapnia-induced increases in respiratory drive (7% CO₂, 21% O₂, balance N₂) (all $P > 0.5$).

To more closely examine the effect of transplantation on specific respiratory circuitry, recordings of phrenic nerve efferent bursting activity (a measure of PhMN output) were obtained from anesthetized graft and lesion-only rats (e.g. Fig. 2F). Arterial PO₂ and PCO₂ values were comparable between experimental groups (data not shown) and within physiologically normal ranges at baseline (Fuller et al., 2008, Fuller et al., 2006). Arterial PO₂ reached 35–40 mmHg during isocapnic hypoxia similar to prior reports (Fuller et al., 2006, Fuller et al., 2003). At baseline, spontaneously recovered inspiratory bursting in the

phrenic nerve ipsilateral (IL) to the hemilesion (Fig. 2F) was observed in all C2Hx (6/6) and FSC_V rats (5/5).

Two lines of evidence suggested a possible differential graft effect. First, recovery of ipsilateral PhMN activity was only seen in 3/5 FSC_D rats (recovered; FSC_{D-R}). In addition, there was a strong tendency for FSC_{D-R} rats to have a blunted response (overall treatment effect $P=0.06$) during respiratory challenge, although no differences were found in integrated phrenic burst amplitude ($\int \text{Phr}$) (Fig. 2G). Respiratory challenge did not induce IL phrenic bursting in the two FSC_D rats in which bursting was absent at baseline. Second, differences in phrenic burst frequency were also noted between the experimental groups. During baseline, burst frequency ($\text{burst} \cdot \text{min}^{-1}$) was lower in FSC_V rats (46 ± 3) compared to FSC_{D-R} (59 ± 3 , $p=0.03$) and C2Hx rats (61 ± 2 , $p=0.03$). Phrenic burst frequency (%baseline) during respiratory challenge was also greater in FSC_V rats during both hypercapnia and hypoxia (Fig. 2H, $P<0.05$).

Contralateral (CL) phrenic neurogram bursting was unaltered by the presence of transplant tissue. All rats showed robust CL phrenic nerve bursting activity (e.g. Fig. 2F). No differences in CL $\int \text{Phr}$ were noted either at baseline (data not shown) or under respiratory challenge conditions (Fig. 2I).

Measurements of mean arterial blood pressure (MAP), obtained during the course of the electrophysiological studies, revealed another difference between experimental groups (Fig. 2J, 2-way RM ANOVA treatment effect $P=0.047$). MAP was significantly lower in FSC_V vs. C2Hx rats during the baseline condition ($p<0.05$). However, no other comparisons revealed significant differences between groups. Finally, all rats showed a decline in MAP during hypoxia (Fig. 2J), as previously reported in C2Hx rats (Fuller et al., 2003). This decline in MAP (%baseline) was similar between groups: FSC_D ($-45 \pm 10\%$), FSC_V rats ($-46 \pm 8\%$), C2Hx ($-33 \pm 12\%$) ($P=0.68$).

Discussion

Spinal circuit reconstruction and establishment of novel neuronal relays are two potential options for promoting functional repair of the injured spinal cord with grafts containing neuronal precursors (Reier, 2004). In that regard, fetal CNS grafting represents a valuable experimental tool that can guide more contemporary stem cell approaches in defining desired transplant characteristics. The present findings provide neuroanatomical evidence for local synaptic integration of donor neurons with an anatomically and functionally characterized host spinal circuitry. To our knowledge, this is the first reported example of such highly defined host-graft neuronal integration coupled with findings suggesting that regionally-derived neural precursor populations can differentially affect functional outcome parameters.

For the present study, we took advantage of the multipotent stem cell composition (Cao et al., 2002, Chow et al., 2000) and extensive differentiative capacity of FSC tissue grafts (Jakeman et al., 1989, Reier et al., 1986) to investigate whether regional derivation of neuronal progenitors would differentially influence respiratory function in a well-established model of neuroplasticity (Goshgarian, 2003). Using SG-like areas as an index of dorsal horn enrichment, the histological contrast between dorsal and ventral FSC transplants is consistent with dorsoventral patterning of neuronal progenitors in the embryonic spinal cord (Jessell, 2000, Lee and Jessell, 1999, Lewis, 2006, Muller et al., 2002). Previous studies have indicated that FSC grafts emerge from glial and neuronal lineage-restricted progenitors that survive donor tissue attrition during the first 4 days after grafting into acute spinal lesions (Lepore and Fischer, 2005, Theele et al., 1996). Therefore, from a developmental

perspective, FSC transplants essentially represent co-grafts of neuronal- and glial-restricted progenitors. The presence of SG-like areas suggests an even greater lineage restriction of some surviving neuronal progenitors. Accordingly, future studies will investigate donor cell expression of post-mitotic transcription factors and homeobox genes known to be associated with gray matter regional differentiation (reviewed in Lewis, 2006).

Electrophysiological recordings suggested that FSC_D recipients had a reduced IL phrenic response to respiratory challenge as illustrated by reductions in both burst amplitude and frequency. The effect on the latter is intriguing, though difficult to explain, since that parameter is regulated by brainstem circuitry with modulation via peripheral afferent inputs. One possibility is a graft interaction with a polysynaptic spinobulbar pathway mediating primary afferent input to supraspinal respiratory centers (Cleland and Getting, 1993) for which supporting neuroanatomical results have been recently described (Lane et al., 2009).

Another observation meriting further investigation was that spontaneous recovery of ipsilateral PhMN activity, which is normally present by 12 weeks post-C2Hx, was absent in 2 of the 5 FSC_D recipients. Though sample size precludes any firm conclusions, the possibility nevertheless exists of an adverse graft impact. Interestingly, these initial FSC_D results are consistent with other lines of evidence for the existence of a population of dorsal horn interneurons that can inhibit PhMN activity via local spinal networks (Zimmer and Goshgarian, 2007). That the differential graft effects seen might not be limited to respiratory responses alone is also indicated by baseline MAP measures in FSC_V recipients.

In contrast to our electrophysiological findings, no graft effect was seen in terms of overall ventilatory behavior which is mediated not only by the ipsilateral phrenic circuit, but also pathways not directly affected by C2Hx (e.g. contralateral phrenic and intercostal). The lack of any noticeable difference in $\dot{V}_E$ across groups, despite evidence of reduced phrenic output in FSC_D rats, may reflect compensatory respiratory mechanisms (Doperalski and Fuller, 2006, Golder et al., 2003) in spared respiratory motor systems which would likely mask changes in the ipsilateral phrenic circuit. Compensatory mechanisms are an important consideration in assessing the therapeutic efficacy of any intervention in models of incomplete spinal cord injury.

Glial-restricted progenitors have been shown to prevent neuronal progenitors from defaulting to an astroglial lineage (Cao et al., 2002) in the non-neurogenic environment of the injured adult spinal cord (Lepore and Fischer, 2005). Accordingly, both glial (e.g., scarring) and neuronal (e.g., total numbers, phenotypic ratios) variables may contribute to the functional changes attributed to the dorsal and ventral FSC grafts, as well as any other form of neuronal replacement strategy. Our PRV data thus provide an important basis for speculating that the neurophysiological effects reported could be associated with some synaptic integration of neurons with the host phrenic circuit (Lane et al., 2008b). However, the post-PRV survival time employed does not allow discrimination between donor neuronal interactions directly with infected PhMNs or indirectly via pre-phrenic interneurons. We also cannot conclude whether the effects of dorsal versus ventral FSC transplants reflect quantitative or qualitative differences in donor neuronal pharmacological/functional subtype or the circuitry of the grafts themselves. Neuronal replacement approaches in the injured spinal cord may not be limited to the notion of programming progenitors to become specific neuronal phenotypes but also definition of intra-transplant connectivity. This principle would hold true for any line of neuronal-restricted donor cells with potentially heterogeneous pharmacological cell fates.

Neuronal replacement has been frequently cited as a possible stem cell-based approach for promoting spinal cord repair. Although therapeutic efficacy was not the immediate focus of

this study, our initial results provide an important indication that some form of local gray matter repair can impact on functional outcomes. Further investigations are needed to test whether respiratory recovery post-SCI can be enhanced by optimizing host-graft axonal interactions as other reports suggest (Lemons et al., 1999, Nikulina et al., 2004).

Because interneurons are essential to the function of neural circuits and regulation of motoneuron excitability (Grillner and Jessell, 2009), other investigations are also needed to define which of the diverse neuronal phenotypes would be optimal for transplantation. Furthermore, there is a need to establish how to best integrate donor cells with desired host substrates in a context-appropriate fashion. In that regard, respiratory neurobiology offers many experimental opportunities for investigating such issues from multidisciplinary perspectives and in relation to a defined spinal circuit in both rats (Lane et al., 2008b) and mice (Qiu et al., 2009). By virtue of their predominant, if not exclusive interneuronal composition, FSC grafts, combined with genetic manipulation (Goulding, 2009), offer important experimental opportunities to assess potential repair contributions of interneuronal subtypes based on well-established developmental principles.

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References

- Bonner JF, Blesch A, Neuhuber B, Fischer I. Promoting directional axon growth from neural progenitors grafted into the injured spinal cord. *J. Neurosci. Res* 2010;88:1182–1192. [PubMed: 19908250]
- Cao Q, He Q, Wang Y, Cheng X, Howard RM, Zhang Y, Devries WH, Shields CB, Magnuson DS, Xu XM, Kim DH, Whittemore SR. Transplantation of Ciliary Neurotrophic Factor-Expressing Adult Oligodendrocyte Precursor Cells Promotes Remyelination and Functional Recovery after Spinal Cord Injury. *J. Neurosci* 2010;30:2989–3001. [PubMed: 20181596]
- Cao QL, Howard RM, Dennison JB, Whittemore SR. Differentiation of engrafted neuronal-restricted precursor cells is inhibited in the traumatically injured spinal cord. *Exp. Neurol* 2002;177:349–359. [PubMed: 12429182]
- Chow SY, Moul J, Tobias CA, Himes BT, Liu Y, Obrocka M, Hodge L, Tessler A, Fischer I. Characterization and intraspinal grafting of EGF/bFGF-dependent neurospheres derived from embryonic rat spinal cord. *Brain Res* 2000;874:87–106. [PubMed: 10960593]
- Cizkova D, Kakinohana O, Kucharova K, Marsala S, Johe K, Hazel T, Hefferan MP, Marsala M. Functional recovery in rats with ischemic paraplegia after spinal grafting of human spinal stem cells. *Neuroscience* 2007;147:546–560. [PubMed: 17524565]
- Cleland CL, Getting PA. Respiratory-modulated and phrenic afferent-driven neurons in the cervical spinal cord (C4–C6) of the fluorocarbon-perfused guinea pig. *Exp. Brain Res* 1993;93:307–311. [PubMed: 8491269]
- Doperalski NJ, Fuller DD. Long-term facilitation of ipsilateral but not contralateral phrenic output after cervical spinal cord hemisection. *Exp. Neurol* 2006;200:74–81. [PubMed: 16647702]
- Eftekharpour E, Karimi-Abdolrezaee S, Fehlings MG. Current status of experimental cell replacement approaches to spinal cord injury. *Neurosurg. Focus* 2008;24:E19. [PubMed: 18341395]
- Fuller DD, Doperalski NJ, Dougherty BJ, Sandhu MS, Bolser DC, Reier PJ. Modest spontaneous recovery of ventilation following chronic high cervical hemisection in rats. *Exp. Neurol* 2008;211:97–106. [PubMed: 18308305]
- Fuller DD, Golder FJ, Olson EB Jr, Mitchell GS. Recovery of phrenic activity and ventilation after cervical spinal hemisection in rats. *J. Appl. Physiol* 2006;100:800–806. [PubMed: 16269524]

- Fuller DD, Johnson SM, Olson EB Jr, Mitchell GS. Synaptic pathways to phrenic motoneurons are enhanced by chronic intermittent hypoxia after cervical spinal cord injury. *J. Neurosci* 2003;23:2993–3000. [PubMed: 12684486]
- Fuller DD, Sandhu MS, Doperalski NJ, Lane MA, White TE, Bishop MD, Reier PJ. Graded unilateral cervical spinal cord injury and respiratory motor recovery. *Respir. Physiol. Neurobiol* 2009;165:245–253. [PubMed: 19150658]
- Golder FJ, Fuller DD, Davenport PW, Johnson RD, Reier PJ, Bolser DC. Respiratory motor recovery after unilateral spinal cord injury: eliminating crossed phrenic activity decreases tidal volume and increases contralateral respiratory motor output. *J. Neurosci* 2003;23:2494–2501. [PubMed: 12657710]
- Goldman SA, Windrem MS. Cell replacement therapy in neurological disease. *Philos. Trans. R Soc. Lond. B Biol. Sci* 2006;361:1463–1475. [PubMed: 16939969]
- Goshgarian HG. The crossed phrenic phenomenon: a model for plasticity in the respiratory pathways following spinal cord injury. *J Appl Physiol* 2003;94:795–810. [PubMed: 12531916]
- Goulding M. Circuits controlling vertebrate locomotion: moving in a new direction. *Nat. Rev. Neurosci* 2009;10:507–518. [PubMed: 19543221]
- Grillner S, Jessell TM. Measured motion: searching for simplicity in spinal locomotor networks. *Curr. Opin. Neurobiol* 2009;19:572–586. [PubMed: 19896834]
- Hadi B, Zhang YP, Burke DA, Shields CB, Magnuson DS. Lasting paraplegia caused by loss of lumbar spinal cord interneurons in rats: no direct correlation with motor neuron loss. *J. Neurosurg* 2000;93:266–275. [PubMed: 11012058]
- Hooshmand MJ, Sontag CJ, Uchida N, Tamaki S, Anderson AJ, Cummings BJ. Analysis of host-mediated repair mechanisms after human CNS-stem cell transplantation for spinal cord injury: correlation of engraftment with recovery. *PLoS ONE* 2009;4:e5871. [PubMed: 19517014]
- Jakeman LB, Reier PJ. Axonal projections between fetal spinal cord transplants and the adult rat spinal cord: a neuroanatomical tracing study of local interactions. *J. Comp. Neurol* 1991;307:311–334. [PubMed: 1713233]
- Jakeman LB, Reier PJ, Bregman BS, Wade EB, Dailey M, Kastner RJ, Himes BT, Tessler A. Differentiation of substantia gelatinosa-like regions in intraspinal and intracerebral transplants of embryonic spinal cord tissue in the rat. *Exp. Neurol* 1989;103:17–33. [PubMed: 2912747]
- Jessell TM. Neuronal specification in the spinal cord: inductive signals and transcriptional codes. *Nat. Rev. Genet* 2000;1:20–29. [PubMed: 11262869]
- Karimi-Abdolrezaee S, Eftekharpour E, Wang J, Schut D, Fehlings MG. Synergistic effects of transplanted adult neural stem/progenitor cells, chondroitinase, and growth factors promote functional repair and plasticity of the chronically injured spinal cord. *J. Neurosci* 2010;30:1657–1676. [PubMed: 20130176]
- Kim BG, Dai HN, Lynskey JV, McAtee M, Bregman BS. Degradation of chondroitin sulfate proteoglycans potentiates transplant-mediated axonal remodeling and functional recovery after spinal cord injury in adult rats. *J. Comp. Neurol* 2006;497:182–198. [PubMed: 16705682]
- Kocsis JD, Akiyama Y, Radtke C. Neural precursors as a cell source to repair the demyelinated spinal cord. *J. Neurotrauma* 2004;21:441–449. [PubMed: 15115593]
- Lane MA, Fuller DD, White TE, Reier PJ. Respiratory neuroplasticity and cervical spinal cord injury: translational perspectives. *Trends Neurosci* 2008a;31:538–547. [PubMed: 18775573]
- Lane MA, Lee KZ, Fuller DD, Reier PJ. Spinal circuitry and respiratory recovery following spinal cord injury. *Respir. Physiol. Neurobiol.* 2009
- Lane MA, White TE, Coutts MA, Jones AL, Sandhu MS, Bloom DC, Bolser DC, Yates BJ, Fuller DD, Reier PJ. Cervical prephrenic interneurons in the normal and lesioned spinal cord of the adult rat. *J. Comp. Neurol* 2008b;511:692–709. [PubMed: 18924146]
- Lee KJ, Jessell TM. The specification of dorsal cell fates in the vertebrate central nervous system. *Ann. Rev. Neurosci* 1999;22:261–294. [PubMed: 10202540]
- Lemons ML, Howland DR, Anderson DK. Chondroitin sulfate proteoglycan immunoreactivity increases following spinal cord injury and transplantation. *Exp. Neurol* 1999;160:51–65. [PubMed: 10630190]

- Lepore AC, Fischer I. Lineage-restricted neural precursors survive, migrate, and differentiate following transplantation into the injured adult spinal cord. *Exp. Neurol* 2005;194:230–242. [PubMed: 15899260]
- Lewis KE. How do genes regulate simple behaviours? Understanding how different neurons in the vertebrate spinal cord are genetically specified. *Philos. Trans. R Soc. Lond. B Biol. Sci* 2006;361:45–66. [PubMed: 16553308]
- Magnuson DS, Lovett R, Coffee C, Gray R, Han Y, Zhang YP, Burke DA. Functional consequences of lumbar spinal cord contusion injuries in the adult rat. *J. Neurotrauma* 2005;22:529–543. [PubMed: 15892599]
- Mitsui T, Shumsky JS, Lepore AC, Murray M, Fischer I. Transplantation of neuronal and glial restricted precursors into contused spinal cord improves bladder and motor functions, decreases thermal hypersensitivity, and modifies intraspinal circuitry. *J. Neurosci* 2005;25:9624–9636. [PubMed: 16237167]
- Muller T, Brohmann H, Pierani A, Heppenstall PA, Lewin GR, Jessell TM, Birchmeier C. The homeodomain factor *lhx1* distinguishes two major programs of neuronal differentiation in the dorsal spinal cord. *Neuron* 2002;34:551–562. [PubMed: 12062039]
- Nikulina E, Tidwell JL, Dai HN, Bregman BS, Filbin MT. The phosphodiesterase inhibitor rolipram delivered after a spinal cord lesion promotes axonal regeneration and functional recovery. *Proc. Natl. Acad. Sci. U S A* 2004;101:8786–8790. [PubMed: 15173585]
- Olson EB Jr, Bohne CJ, Dwinell MR, Podolsky A, Vidruk EH, Fuller DD, Powell FL, Mitchel GS. Ventilatory long-term facilitation in unanesthetized rats. *J. Appl. Physiol* 2001;91:709–716. [PubMed: 11457785]
- Qiu, K.; Lane, MA.; Reier, PJ.; Fuller, DD. *Exper. Biol. Meeting. New Orleans: 2009.* Neuroanatomical characterization of the phrenic nucleus in adult mice. Abstract 783.782
- Reier PJ. Cellular transplantation strategies for spinal cord injury and translational neurobiology. *NeuroRx* 2004;1:424–451. [PubMed: 15717046]
- Reier PJ, Bregman BS, Wujek JR. Intraspinal transplantation of embryonic spinal cord tissue in neonatal and adult rats. *J. Comp. Neurol* 1986;247:275–296. [PubMed: 3522658]
- Sasaki M, Li B, Lankford KL, Radtke C, Kocsis JD. Remyelination of the injured spinal cord. *Prog. Brain Res* 2007;161:419–433. [PubMed: 17618995]
- Sharp J, Frame J, Siegenthaler M, Nistor G, Keirstead HS. Human embryonic stem cell-derived oligodendrocyte progenitor cell transplants improve recovery after cervical spinal cord injury. *Stem Cells* 2010;28:152–163. [PubMed: 19877167]
- Sieradzian K, Vrbova G. Replacement of missing motoneurons by embryonic grafts in the rat spinal cord. *Neuroscience* 1989;31:115–130. [PubMed: 2771053]
- Teng YD, Mocchetti I, Taveira-DaSilva AM, Gillis RA, Wrathall JR. Basic fibroblast growth factor increases long-term survival of spinal motor neurons and improves respiratory function after experimental spinal cord injury. *J. Neurosci* 1999;19:7037–7047. [PubMed: 10436058]
- Theele DP, Schrimsher GW, Reier PJ. Comparison of the growth and fate of fetal spinal iso- and allografts in the adult rat injured spinal cord. *Exp. Neurol* 1996;142:128–143. [PubMed: 8912904]
- Vierck CJ Jr, Siddall P, Yezierski RP. Pain following spinal cord injury: animal models and mechanistic studies. *Pain* 2000;89:1–5. [PubMed: 11113287]
- Yezierski RP, Liu S, Ruenes GL, Kajander KJ, Brewer KL. Excitotoxic spinal cord injury: behavioral and morphological characteristics of a central pain model. *Pain* 1998;75:141–155. [PubMed: 9539683]
- Zimmer MB, Goshgarian HG. GABA, not glycine, mediates inhibition of latent respiratory motor pathways after spinal cord injury. *Exp. Neurol* 2007;203:493–501. [PubMed: 17046753]

Figure 1.

A. Schematic diagram of the tissue microdissection procedure employed. A rostro-caudal incision was made along the midline of spinal cord separating it into two halves (**Ai**). From this, the dorsal- and ventral-most parts of the tissue (from the alar and basal plates, respectively) were then removed to obtain strips of tissue for transplantation (**Aii**, FSC_D and FSC_V). **B.** Transverse sections (2μm) of adult spinal cord stained with toluidine blue, at the lesion/transplant epicenter 2 months post-C2Hx. These images demonstrate the growth of transplanted tissue (left half of each section). **C.** Higher magnification images reveal that FSC transplants retain some developmental cues, with the formation of clusters of small neurons into myelin-free, SG-like regions (SG) in FSC_D, and the presence of large bands of

white matter (arrowheads) throughout FSC_V. Measurement of the proportion of SG-like regions ($\pm$ SD) in these animals confirms a significantly greater prevalence in FSC_D recipients. **D.** Transverse sections (40 μ m) immunolabeled for NeuN demonstrates the large number of mature neurons throughout the graft in both FSC_D and FSC_V recipients. These sections also show the similarity between the clusters of small neurons into SG-like regions (SG) and the host SG (SG_H). **E.** In a subset of animals the phrenic circuit ipsilateral to C2Hx was retrogradely traced with a transynaptic tracer (PRV). Longitudinal sections of spinal cord at the level of the ventral horns showing host and transplant. The dotted line is an approximation of the interface between host and graft tissue. Immunolabeling for PRV reveals labeled phrenic motoneurons (PhMN) and cells within FSC_D and FSC_V transplants. This suggests that donor neurons become synaptically integrated with the host phrenic circuit and provides an anatomical basis for how these cells may influence ipsilateral phrenic activity.

Figure 2.

A. Representative examples of airflow traces recorded in unanesthetized rats and used to derive breathing frequency (F), tidal volume (TV) and minute ventilation ($\dot{V}_E$). Two different time scales are provided - the arrows in the expanded traces point to individual breaths indicated by downward traces (i.e. negative airflow). Scaling is the same in the Pre-C2Hx and 12 wk post-C2Hx traces; airflow is in units of ml/sec. Note the rapid, shallow pattern of breathing during the baseline condition after C2Hx as indicated by the increase in breathing frequency and the reduction in airflow. The mean baseline F (**B**), TV (**C**), and $\dot{V}_E$ (**D**) responses to injury were not different between C2Hx, FSC_V and FSC_D rats nor were there any differences in $\dot{V}_E$ during respiratory challenge (**E**). Examples of efferent phrenic bursting recorded in anesthetized rats at 12 wks post-injury are provided in panel F. The arrows indicate relatively weak bursting at baseline in the FSC_V rat. There was a tendency for ipsilateral (IL) $\int$ Phr amplitude to be reduced in FSC_V rats during challenge ($P=0.06$, **G**). Phrenic burst frequency (F) was enhanced in FSC_V rats (**H**). Contralateral (CL) Phr was similar between groups (**I**). Mean arterial pressure (MAP) was reduced in FSC_V rats during baseline (**J**). Symbols: ■-FSC_V, ◆-FSC_D, ●-C2Hx, ↓O₂-hypoxia, ↑CO₂-hypercapnia