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A re-assessment of long distance growth and connectivity of neural stem cells after severe spinal cord injury

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ABSTRACT

As part of the NIH "Facilities of Research Excellence—Spinal Cord Injury" project to support independent replication, we repeated key parts of a study reporting robust engraftment of neural stem cells (NSCs) treated with growth factors after complete spinal cord transection in rats. Rats (n = 20) received complete transections at thoracic level 3 (T3) and 2 weeks later received NSC transplants in a fibrin matrix with a growth factor cocktail using 2 different transplantation methods (with and without removal of scar tissue). Control rats (n = 9) received transections only. Hindlimb locomotor function was assessed with the BBB scale. Nine weeks post injury, reticulospinal tract axons were traced in 6 rats by injecting BDA into the reticular formation. Transplants grew to fill the lesion cavity in most rats although grafts made with scar tissue removal had large central cavities. Grafts blended extensively with host tissue obliterating the astroglial boundary at the cut ends, but in most cases there was a well-defined partition within the graft that separated rostral and caudal parts of the graft. In some cases, the partition contained non-neuronal scar tissue. There was extensive outgrowth of GFP labeled axons from the graft, but there was minimal ingrowth of host axons into the graft revealed by tract tracing and immunocytochemistry for 5HT. There were no statistically significant differences between transplant and control groups in the degree of locomotor recovery. Our results confirm the previous report that NSC transplants can fill lesion cavities and robustly extend axons, but reveal that most grafts do not create a continuous bridge of neural tissue between rostral and caudal segments.

Introduction

In recent years, there have been numerous reports of interventions that enhance sparing of function and/or promote repair mechanisms including axon regeneration so as to enhance recovery of function after spinal cord injury. A barrier to translation is that there are often no published replications of promising studies, raising questions about reproducibility. To address this, the National Institute of Neurological Disorders and Stroke (NINDS) launched the "Facilities of Research Excellence—Spinal Cord Injury" (FORE-SCI) replication project, in which promising published studies are independently replicated. Studies are selected for replication by an independent scientific advisory committee and executed by one of the sites funded by the FORE-SCI Project. Here, we repeat an experiment that reported remarkable outgrowth of axons from transplants of neural stem cells (NSCs) that were treated with a cocktail of growth factors and transplanted into the lesion site after complete transection of the spinal cord (Lu et al., 2012).

The study of Lu et al. was based on previous evidence that regeneration failure after CNS injury was due to a combination of factors including myelin-derived inhibitors (Buchli and Schwab, 2005; He and Koprivica, 2004), inhibitory molecules expressed by reactive astrocytes near the injury site (Busch and Silver, 2007; Fitch and Silver, 2008), and lack of intrinsic growth capacity (Jones et al., 2001), for a recent review, see (Tuszynski and Steward, 2012). Previous work demonstrating axon regeneration through peripheral nerve grafts in the CNS documents that adult neurons can regenerate their axons to some extent when provided with an optimal tissue environment (David and Aguayo, 1981; Houle et al., 2006), but growth is limited due to neuron-intrinsic mechanisms (Filbin, 2006; Liu et al., 2010). The study of Lu et al. was based on the commonly held belief that a combinational treatment would be most effective. The specific approach was to transplant freshly dissociated neural stem cells/progenitors from rat embryos as in previous studies (Lepore and Fischer, 2005), but pre-treating cells with a growth factor cocktail and transplanting with a fibrin matrix. The injury model was a complete transection at thoracic level 3 (T3). Complete spinal cord transections are considered the most stringent test of regenerative strategies (Tuszynski and Steward, 2012).

We repeated key experiments in the original study to assess reproducibility, focusing on the portion of the report that used freshly

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isolated NSCs from fetal rats. Our results confirm that NSC transplants engraft and differentiate, although the extent of engraftment was variable and depended on the method of transplant. Our results also confirm remarkable axon outgrowth from NSC transplants. There was only limited ingrowth of host axons, however. Assessments of locomotor function did not reveal statistically significant differences between transplanted and control groups. A completely unexpected finding was that in about 50% of the rats, ectopic masses of graft-derived cells were found at long distances from the transplant site including in the brain. We report the existence of these ectopic colonies separately (Steward et al., 2014). Our findings identify important issues relating to the potential use of NSC transplants for therapy for severe SCI, including a need to further refine transplantation techniques.

Materials and methods

Details regarding the way that the FORE-SCI Replication contracts operate are summarized in perfect Steward et al., 2012. In brief, an independent Scientific Advisory Committee for the contract reviews the literature and selects papers for replication. Selection factors include: 1) Clinically-relevant endpoints (usually recovery of function); 2) Translatability; 3) Effect size; and 4) Scientific quality of the target paper. The Committee may select only part of the published study for replication (a particular set of experiments), and may recommend additional control groups or analyses. Experiments that were not part of the original paper, including additional control groups, are not supported by the Contract unless recommended by the Scientific Advisory Committee. Here, the Committee selected the portion of Lu et al. (2012) involving transplants of rat NSCs, with assessment of engraftment, axon outgrowth from the graft, ingrowth of host axons, and assessment of locomotor function.

Experimental animals were female Fischer 344 rats from Harlan Labs that were 10–11 weeks old at the start of the experiments (the same strain, sex, and age used by Lu et al.). Experimental protocols on spinal cord injured rats were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Irvine. Rats genetically engineered to express EGFP (F344-Tg(UBC-EGFP)F455Rrrc) were obtained from the Rat Resource & Research Center (P40D011062) and were bred locally to obtain embryos in the animal facilities at the Veteran's Hospital San Diego. Procedures used to collect fetal NSCs were approved by the IACUC at the Veteran's Hospital San Diego.

Every effort was made to duplicate procedures used by Lu et al. In this regard, Dr. Lu consulted on the procedures and trained our surgical staff. Because this is a surgical intervention, and as such may depend on skills that can only be mastered by extensive experience, we felt that the goals of the replication would be best-served if the same surgeon actually performed the transplants. Accordingly, Dr. Lu traveled to UCI to perform the surgical procedures with the aid of our staff. Also, to insure comparability of NSC transplants, the cells were harvested and prepared in Dr. Lu's lab at the Veterans Administration Medical Center, San Diego (VAMC) and were delivered to UCI on the day of the grafting procedure. We felt that this would be consistent with the goals of the replication because the cells could be considered to be a "product" that was obtained for use as a therapeutic agent in much the same way as a drug from a commercial entity.

Overall experimental plan and group formulation

In the original study, transplantation surgeries were performed on 5–6 rats a day generating a total of 55 rats with full transection injuries. There was no description in the original publication of how surgeries were distributed over time, how rats were assigned to groups and whether group assignment was random.

In our replication, rats were randomly assigned to 2 groups at the time of the transection surgery. A study member who was not involved in the behavioral assessment used a random generator to determine the order of surgery and the group assignments. A) one group was designated to receive transections only. B) one group was designated to receive transections and then grafts 2 weeks later. It is important to note that although the control group (A) did not receive any treatment at the time rats in Group B received grafts, they were anesthetized and received a skin incision that was closed with staples so that the experimenters could remain blind as to group identity. Six rats that received transections and grafts received BDA injections into the reticular formation; grafted animals were randomly chosen from each squad using a random number generator. Rats that received transections and grafts and exhibited recovery of locomotor function received a secondary full transection rostral to the original graft site 6 weeks post-transplant. The schedule of surgeries is summarized in Table 1.

For Squad 1 ($n = 6$) the transection surgery sequence by treatment group was B, B, B, B, A, B. The same group's sequence for transplantation was B, B, A, B, B and B. Four of the six rats received grafts with removal of scar tissue (Method 1, see below); one rat received 9-point injections without scar removal (Method 2).

For Squad 2 ($n = 6$) the transection surgery sequence by treatment group was A, B, B, B, B, B the same group's transplantation sequence was B, B, B, B, B and A. Grafts were done with scar removal (Method 1).

For Squad 3 ($n = 9$) the transection surgery sequence by treatment group was A, B, A, B, A, A, B, B, B and the group's transplantation sequence was B, A, B, A, A, B, B, B, A. Grafts were done using the 9-point injections without scar removal (Method 2).

For Squad 4 ($n = 8$) the transection sequence by treatment group was B, A, B, B, A, A, B, B and the group's transplantation sequence was B, A, B, A, B, B, B, A. Grafts were done using the 9-point injections without scar removal (Method 2).

Spinal cord injury

Rats were anesthetized with ketamine (67 mg/kg) and xylazine, (0.7 mg/kg) and placed on a platform to eliminate movement. Respiratory pattern was monitored throughout the entire duration of the surgery. A laminectomy was performed at thoracic level 3 (T3) and a longitudinal 2 mm cut was made in the dura mater with a #11 blade. Using irridectomy scissors (Roboz, Gaithersburg, MD: cat # RS-5619), a 1.0–1.5 mm longitudinal block of spinal cord was cut on the right side and removed by aspiration with a 23 G blunt needle leaving a 1.0–1.5 mm gap. The same procedures were performed on the left completing the full transection and the site was visually inspected under a dissecting microscope to ensure that the transection was complete ventrally and laterally. Care was taken to preserve the dura mater so that transplanted cells would be confined within the lesion cavity, and to spare dorsal roots. After achieving hemostasis, overlying muscles were sutured with 4–0 silk (Henry Schien, Dublin, Ohio, 100-6830) and NeoPredef antibiotic powder (Western Medical Supply, Arcadia, CA, 94030) was applied, then the skin was closed using wound clips (Fisher Scientific, Pittsburg, PA).

Animal care

Following surgery, rats were placed in cages on water circulating jacketed heating pads for the duration of the study. After recovering from the anesthetic, animals were housed 3–4 per cage. After surgery,

Table 1
Schedule for all surgical procedures.

Squad	T3 transection date	Transplant date	BDA date	Re-transection date
1	4/23/13	5/7/13	6/18/13	6/18/13
2	4/26/13	5/9/13		6/25/13
3	4/30/13	5/14/13	6/28/13	
4	5/2/13	5/16/13	6/28/13	