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A re-assessment of a combinatorial treatment involving Schwann cell transplants and elevation of cyclic AMP on recovery of motor function following thoracic spinal cord injury in rats

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ABSTRACT

This study was undertaken as part of the NIH "Facilities of Research-Spinal Cord Injury" project to support independent replication of published studies. Here, we repeated a study reporting that a combinatorial treatment with transplants of Schwann cells, systemic delivery of Rolipram to enhance cyclic AMP levels, and intra-spinal injections of dibutyryl cyclic AMP enhanced locomotor recovery in rats after contusion injuries at the thoracic level. We compared the following experimental groups: 1) rats that received Schwann cell transplants, systemic Rolipram, and injections of db-cyclic AMP (the combined treatment group that showed the greatest improvement in function); 2) rats that received Schwann cell transplants only and implantation of empty pumps as control; 3) rats that received Rolipram only and implantation of empty pumps as control, and 4) control rats that received no treatment other than the injection of DMEM into the spinal cord and implantation of empty pumps. The principal findings reported in Pearse et al. were *not* replicated in that the combined treatment group did not exhibit greater recovery on any of the measures, although the group that received Schwann cells only did exhibit enhanced recovery on several of the outcome measures. The failure of the combined treatment may be due in part to less successful engraftment of Schwann cells in our study vs. Pearse et al. Issues relating to failures to replicate, especially when effect size is small, are discussed.

Introduction

In recent years, there have been numerous reports of interventions that either enhance sparing of function and/or promote repair mechanisms including axon regeneration so as to enhance recovery of function after spinal cord injury. Although a number of strategies show promise for enhancing recovery, a barrier to translation is that promising findings are often not re-evaluated in independent replications to assess the robustness and reproducibility of the effects. To meet this need, the 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 in which rats that received a combinatorial treatment of transplants of Schwann cells, systemic delivery Rolipram to enhance cyclic AMP levels, and intra-spinal injections of dibutyryl cyclic AMP exhibited enhanced locomotor recovery after contusion injuries at the thoracic level (Pearse et al., 2004).

The original study was based on the general rationale that numerous interventions seem to produce incremental improvements in regenerative growth and/or recovery of function, and so combining different interventions might produce additive or even multiplicative beneficial effects (a combinatorial approach). The specific interventions were as follows: 1) transplants of Schwann cells into the area of the lesion to promote axon regeneration; 2) treatment with Rolipram®, a phosphodiesterase (PDE) inhibitor, to increase levels of cyclic nucleotides, especially cyclic AMP in the brain and spinal cord; 3) injections of dibutyryl cyclic AMP (db-CAMP) a non hydrolyzable analog of cyclic AMP into the spinal cord near the injury site. Rats received contusion injuries and then each of the individual interventions and interventions in different combinations. Pearse et al. reported that maximal functional improvement (locomotor function) was obtained with the combined treatment with all 3

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interventions. Consistent with the requirements of the FORE-SCI replication project, our goal here was to repeat as closely as possible the key experiments reported in Pearse et al. (2004).

Materials and methods

Planning the replication study was hampered by the lack of details about the exact procedures in the original publication, which is a common problem with papers published in modern high impact journals. The original authors were contacted for clarification of procedural details that were unclear. Despite our concerted effort to do things in the same way, there were some discrepancies in procedures due to misunderstanding. Instances of where our experimental procedures were different from those of the original report are noted below. All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee at the University of California, Irvine.

The original report described results from a total of 8 different groups of animals, with some analyses limited to a subset of the groups. Based on recommendations of the scientific steering committee, we focused on the 4 treatment groups that were seen as representing the key comparison groups. The groups were: 1) rats that received Schwann cell transplants, systemic Rolipram, and injections of db-cyclic AMP (the combined treatment group that showed the greatest improvement in function); 2) rats that received Schwann cell transplants only and implantation of empty pumps as control; 3) rats that received Rolipram only and implantation of empty pumps as control, and 4) control rats that received an injection of DMEM into the spinal cord (a control for Schwann cell transplant surgery) and implantation of empty pumps.

Discussions with the original authors revealed that the overall study was carried out by running different squads of animals at different times over a period of several years. Data from different squads were then combined for graphical presentation and statistical analysis. Running in squads is necessary because of the large number of treatment groups involved, but an important consequence is that different treatment groups are actually not run simultaneously. It was neither practical nor feasible within the constraints of the contract to carry out the experiment in exactly the same way as in the original study (that is, by running small squads of animals over a period of years). Thus, we ran two large squads composed of different experimental groups (called here Squad #1 and Squad #2).

The original authors felt that it was important to obtain the test strain (Fischer rats) from the same supplier that had supplied the rats for the original report because of concerns about possible genetic variability between suppliers or even between the colonies maintained by the same supplier in different locations. Accordingly, rats were purchased from Harlan (Indianapolis, IN). It should be noted that Harlan Lab warrants that rats maintained in different sites do not differ genetically.

To avoid any issues arising from small differences in the methods of preparation of Schwann cells, we obtained Schwann cells from the original authors. Thus, coordinating the replication experiment required that we obtain a sufficient number of rats of the appropriate strain (Fischer), age and sex from a particular supplier (Harlan) and a sufficient number of Schwann cells from Dr. Bunge's lab at the appropriate time for transplantation after SCI. The Schwann cells were then amplified in culture at University of California at Irvine prior to transplantation.

Squad 1

The first squad of rats was run beginning in December 2008. Animals were handled for 2 weeks (a critical step because Fischer rats are emotionally reactive, which complicates functional testing). Thirty two rats then received moderate contusion injuries and implantations of osmotic minipumps on December 15.

Surgical procedures

Rats were anesthetized with ketamine/xylazine (50 mg/kg, 10 mg/kg). When supplemental anesthesia was required, one fourth of the original dose was given. It should be noted that Pearse et al. used 1–2% halothane in a mixture of 70% nitrous oxide and 30% oxygen for anesthesia. Our standard IACUC protocol for spinal cord injury surgery at the time the experiments were done was the ketamine/xylazine mixture, and we felt that this modification in experimental protocol would be inconsequential for the primary outcome measures. Body temperature was maintained by placing rats on a water-circulating jacketed heating pad at 37 ± 0.5 °C. The skin over the upper thoracic area was shaved and cleaned with a Betadyne solution. The skin was incised, and then the connective and muscle tissue were bluntly dissected to expose the vertebrae. A laminectomy was performed involving T8 and half of T9 taking care not to damage the spinal cord during the dorsal lamina removal. Rats were then transferred to the stage of the NYU impactor and received moderate contusion injuries (10 g from 12.5 mm, 2 mm rod diameter) on the exposed dura mater of the spinal cord.

After completion of the contusion injuries, muscles were sutured with 5–0 chromic gut, and the skin was closed with surgical staples. Two rats died during or shortly after surgery due to anesthetic complications. Of the remaining 30 rats, 20 were implanted with two osmotic minipumps that contained Rolipram (Alzet model 2001, Cupertino, CA; delivery rate = 0.5 mg/kg/day); 10 were implanted with two minipumps that were empty (control). The pumps were implanted for 1 week and then exchanged a week later for two more model 2001 osmotic mini-pumps for a total of 14 days of administration.

Three rats were euthanized on the day after surgery because they or their cage mates extracted their implanted osmotic minipumps causing major mutilation to their back skin. The surviving rats ($n = 27$) included 18 in which the osmotic minipumps contained Rolipram and 9 in which the osmotic minipumps were empty.

Post operative care

Following surgery, rats were immediately placed on a water-circulating jacketed heating pad. After recovering from the anesthetic, rats were housed 4–5 per cage. This is highly preferred by our animal care and use committee in order to promote social interactions and animal welfare. It should be noted, however, that rats in Pearse et al. were housed singly (more on this below). For 10–14 days after surgery, rats received daily injections of lactated ringers (5 mg/100 g, sub-cutaneously) for hydration, the analgesic Buprenex (Buprenorphine, 0.01 mg/kg), and Baytril (Enroflaxacin 2.5 mg/kg, sub-cutaneously) for prophylactic treatment against urinary tract infections (UTIs).

Rats were monitored twice daily for general health, coat quality (indicative of normal grooming activity) and mobility within the cage. Rats with moderate contusion injuries typically resume these activities the day following injury. In addition, signs of paralysis were monitored, including lack of hind limb movement, tail flaccidity, and instability/uncoordinated movement. Rats were also monitored for signs of skin lesions on the paralyzed limbs or autophagia of the toes. None of the rats exhibited skin lesions or autophagia throughout the experiment. Bladders were manually expressed twice daily for the entire length of the study. Rats were monitored for urinary tract infections (UTIs) for the entire duration of the experiment and no UTIs were observed.

Our animal care protocol calls for the following: if an animal failed to resume normal activities, showed evidence of skin lesions or