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Allotransplanted DRG neurons or Schwann cells affect functional recovery in a rodent model of sciatic nerve injury

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Abstract

Objective—In this study, the functional recoveries of Sprague-Dawley rats following repair of a complete sciatic nerve transection using allotransplanted dorsal root ganglion (DRG) neurons or Schwann cells were examined using a number of outcome measures.

Methods—Four groups were compared: (1) repair with a nerve guide conduit seeded with allotransplanted Schwann cells harvested from Wistar rats, (2) repair with a nerve guide conduit seeded with DRG neurons, (3) repair with solely a nerve guide conduit, and (4) sham-surgery animals where the sciatic nerve was left intact. The results corroborated our previous reported histology findings and measures of immunogenicity.

Results—The Wistar-DRG-treated group achieved the best recovery, significantly outperforming both the Wistar-Schwann group and the nerve guide conduit group in the Von Frey assay of touch response ($P < 0.05$). Additionally, Wistar-DRG and Wistar-Schwann seeded repairs showed lower frequency and severity in an autotomy measure of the self-mutilation of the injured leg because of neuralgia.

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Disclaimer Statements

Contributors All authors have made a substantial contribution to the concept and design of the study, acquisition of data and analysis, and interpretation of data, and have drafted the article or revised it critically for important intellectual content. All authors approved the version to be published.

Conflicts of interest There are no conflicts of interest.

Ethics approval The experimental protocols performed were approved by the University Committee on Animal Resources of the University of Rochester Medical Center and met the National Institute of Health (NIH) Guidelines for the care and use of laboratory animals.

Conclusion—These results suggest that in complete peripheral nerve transections, surgical repair using nerve guide conduits with allotransplanted DRG and Schwann cells may improve recovery, especially DRG neurons, which elicit less of an immune response.

Keywords

NG conduit tube; Peripheral nerve injury repair; DRG cells; Schwann cells; Functional recovery

Introduction

Each year approximately 360 000 people in the United States suffer a peripheral nerve injury (PNI), which is a leading source of lifelong disability.^{1,2} The most frequent cause of PNIs is motor vehicle accidents, while gunshot wounds, stabbings, and birth trauma are also common factors. Patients suffering from PNI-related disabilities are significant sources of liability to the healthcare system, with average hospital stays of 28 days each year.^{1,2} In recent years, there has been considerable interest in developing new surgical and bioengineering techniques to repair damaged peripheral nerves through transplanted materials of biological or synthetic origin.^{3–6} Although many strategies have been developed to promote axonal regeneration and reinnervation, functional recovery is often incomplete and accompanied by neuropathic pain, which is a major source of morbidity.⁷

The current gold standard of treatment for nerve transection is the use of autologous nerve graft.^{8–11} When compared to other techniques such as allotransplantation or bioengineered nerve guide conduits, autografts have been established as the most reliable choice, significantly outperforming alternatives in all measures of motor function.¹² Regeneration of nerves requires a structural scaffold that supports axonal growth with both a non-immunogenic environment and the appropriate microarchitecture. Autografts satisfy both requirements as they are typically available in the host and create immediate direct contact between separated nerve segments.⁸ Complications arise, however, because of the limited supply of donor nerves and the risks associated with the harvesting surgery. Donor sites are vulnerable to infections, the formation of painful neuromas, and loss of function associated with the harvested nerve.

In humans, nerve conduits have been shown to be a viable option for repairs of short nerve lesions and are typically used for gaps ,2–3 cm.^{5,6,13} Conduits are attractive because they can isolate growth paths, thus blocking neuroma formation and infiltration of other tissues while containing Schwann cells and neurotrophic factors that facilitate functional recovery.⁷ Following nerve transection, the axons in the distal stump lose connection to their cell bodies and disintegrate in a process termed Wallerian degeneration.¹⁴ But Schwann cells undergo significant phenotypic modulation to regain the ability to migrate and proliferate, regulating the environment of the injury site. They produce factors that control Wallerian degeneration, counteract inflammatory cytokines, and promote axon regeneration.¹⁵ They also clear the axon debris left by the initial breakdown.¹⁶ Manipulating Schwann cells by activating dormant cells or by injecting progenitor cells has been shown to enhance axon regeneration.⁷ It has been suggested that supplementing allografts with Schwann cells would be of great clinical use in long-gap repairs.¹⁴

The importance of Schwann cells during repair has been evident in studies where their absence is associated with reduced chance of functional recovery.⁷ Chronic denervation of Schwann cells actually leads to the up-regulation of proteoglycans and other factors that mitigate regeneration.¹⁷ This is potentially an underlying factor of neuropathic pain.¹⁵

However, Schwann cells present the challenge of being immunogenic, expressing major histocompatibility complex class I (MHC I) proteins. Neurons, on the other hand, are one of the few cells that do not present MHC I. Dorsal root ganglion (DRG) neurons demonstrate great ability to spontaneously regenerate following PNI,¹⁸ and following axotomy, express growth factors supporting axon regeneration.¹⁶ Elongated DRG neurons used to repair injuries have exhibited long-term survival in the absence of immunosuppressive therapy.¹⁹

The technique of seeding nerve guide conduits with neurons is a promising therapy. In a study by Cullen *et al.*, allogenic DRG neurons seeded in conduits showed a robust outgrowth of axons, demonstrating the possibility of replacing injured neurons across long distances without autografts.²⁰ In a rat model, our group studied the immunogenicity of conduits seeded with DRG cells.²¹ We also tested conduits seeded with allotransplanted Schwann cells to see if they supported an environment more conducive to axon growth.²¹ We found elevated levels of serum IFN-gamma and macrophages in the conduits seeded with Schwann cells, leading to the conclusion that DRG cell-seeded conduits presented as a better option.²¹

However, histologic results do not always correlate well with functional outcomes.²² Therefore, this study expands upon our prior study (Liu *et al.*²¹) and evaluates the functional recoveries of Schwann and DRG cell-seeded nerve conduits presented in those rats, evaluating sensory and motor recovery as well as the resolution of neuropathic pain. Nerve injuries frequently result in chronic and debilitating pain,²³ and many studies have explored treatments that would eliminate or attenuate that pain.^{24–26} In PNI, successful regeneration of distal axons may lead to resolution of pain,²⁷ but other cases have shown that pain is not reduced despite recovery of function.²⁶ To address this concern, we evaluated a number of measures, including autotomy – the self-mutilation of the limb as a response to pain – and a commonly used model to evaluate neuralgia.^{28,29}

Methods

A total of 22 adult male Sprague-Dawley rats (Charles River, Wilmington, MA, USA) were used in this study. The DRG and Schwann cells used were harvested and cultured from Wistar rat pups (Charles River, Wilmington, MA, USA) as detailed in our prior study.²¹ The experimental protocols performed were approved by the University Committee on Animal Resources of the University of Rochester Medical Center, and met the National Institute of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Surgery

The Sprague-Dawley rats were randomly assigned to four treatment groups: in the Wistar-Schwann group ($n = 6$), the sciatic nerve transection was repaired with a NeuraGen® nerve guide conduit (2.0 mm inside diameter $\times$ 1.0 cm length; Integra LifeSciences Corp,

Plainsboro, NJ, USA) seeded with $\sim 1.5 \times 10^6$ Schwann cells; in the Wistar-DRG group ($n = 5$), the transection was repaired with the same conduit, but seeded with $\sim 1.5 \times 10^6$ DRG cells; in the nerve guide group (NG, $n = 7$), transection was repaired with an empty conduit; and in the sham-operated control group (Sham, $n = 4$), the sciatic nerve was exposed but not transected.

Surgical procedures followed those described and depicted by Liu *et al.*²¹ Rats were given buprenorphine analgesia and anesthetized with ketamine (100 mg/kg, i.p.). Once under deep anesthesia, a 1.5 cm skin incision was made in the left upper thigh. The gluteal muscle was dissected to expose the sciatic nerve above its trifurcation. At this point, 8 mm of nerve was excised from rats in the Wistar-Schwann, Wistar-DRG, and NG groups, and the nerve guide conduit was sutured in to bridge the gap between the distal and proximal stumps. Wistar-Schwann and Wistar-DRG conduits were filled with their respective allotransplanted cells. Incisions were sutured closed and disinfected. Post-surgery, all rats received the same treatment: 12-h light–dark cycle, and food and water ad libitum.

Outcome Measures

A number of measures were taken to compare innervation, the recovery of sensory and motor function, and the severity of pain between groups. All recordings were conducted 16 weeks post-surgery by a blinded investigator. Except for the von Frey assay, there were no baseline tests done at the start of the experiment, and thus it is assumed that there were no inherent differences between legs (e.g. skin integrity, muscle bulk, and tone). In the weeks before recording, rats were handled to acclimatize them to testing conditions.

Skin temperature

In a study conducted by Wakisaka³⁰, rats with sciatic nerve injury presented abnormal cutaneous temperature differences between the hind paws, suggesting poor thermal regulation because of abnormal innervation. In normal rats, the temperature difference between paws was insignificant, but with nerve injury, 75% of rats had significant differences between the paws of the intact and injured limbs. The temperatures of the injured paws were highly variable, fluctuating from abnormally hot to abnormally cold.³⁰ In this experiment, animals were wrapped in a surgical towel during testing. Both paws were measured using an infrared laser thermometer (Braintree Scientific, Braintree, MA, USA). Three temperatures were taken in areas devoid of fur and averaged. The absolute percentage difference of the temperature of the injured side from that of the intact side was used in the analysis, with no discrimination made between those injured-side temperatures above intact-side temperatures and those below.

Hot plate test

A measure of pain because of external stimulus, the hot plate test, is commonly used to evaluate the return of sensory function.¹⁶ A hot plate (EchoTherm™ Model IC20XR, Torrey Pines Scientific, Carlsbad, CA, USA) was stabilized at 55°C before testing. A rat draped in a surgical towel was allowed to voluntarily step on the hot surface while supported. Withdrawal time was measured with a stopwatch by a blinded investigator. Normal response

is typically less than 3 seconds. If a response was not observed within 10 seconds, the investigator removed the rat to avoid tissue damage. Three measures were taken per foot. The percentage difference of the injured-side latency from the intact-side latency was recorded.

Extensor postural thrust (EPT)

Extensor postural thrust has been demonstrated to be a consistent measure of motor recovery in rat sciatic nerve transection models.²² Here, the methods established by Thalhammer *et al.* were used.³¹ The rat was held upright in a surgical towel so that the body weight was supported by the distal metatarsals and toes (Fig. 1). The force applied by the hind paws was measured using a digital scale (VIC-1501, Acculab). Three measurements each were taken for both left and right legs. The averaged values were converted to a percentage difference of the injured limb from the intact limb.

Von Frey assay

There is prevalent use of von Frey hair stimulation to quantitatively measure threshold sensory responses.³² However, in this study, a consistent display of normal sensory response was valued over a threshold response, which can register responses to greater forces, but inconsistently. So instead of using a series of hairs, a repetitive measurement with a single hair was used. A 55-g filament was chosen as the threshold force after it produced 100% responses in 10 attempts on both hind paws before surgery. In testing, rats were placed on a wire mesh platform and enclosed in a transparent plexiglass cubicle (Fig. 2). The von Frey hairs (Ugo Basile, Varese, Italy) were introduced through the wire mesh and kept in contact with the plantar surface for 3 seconds. Paw withdrawal to the stimulus within this timeframe was taken as a positive response. During testing, each paw was tested with the 55-g filament 10 times, and the percentage of positive responses was recorded. Only rats whose injured paw demonstrated 100% positive responses were considered to have a return of normal sensory response, and the analysis separated these from rats with any deficiencies.

Autotomy

When the hind limb is denervated, there is a tendency for the rat to self-mutilate the hind paw.^{28,29} There is strong evidence that the self-mutilation is because of painful sensations parallel to those experienced by human amputees. The pain is long-lasting, so there is great interest in pain relief accompanying functional recoveries.²⁸ Here autotomy was scored using the 0–11 scale introduced by Wall *et al.*²⁹ The removal of one or more nails received a score of 1. Each mutilated distal-half digit added an additional point, as did each mutilated proximal-half digit.²⁹

Statistical Analysis

All statistical analyses were conducted in R (version 2.12.0) using a significance level of $\alpha = 0.05$.

Skin temperature, hot plate test, and EPT

Any significant effect that incision surgery had on function was measured by comparing Sham group intact and injured legs in an unpaired Student's *t*-test. Equal variance was assumed. Following a one-way ANOVA, Tukey's HSD (honestly significant difference) *post hoc* assessments of all four treatment groups were conducted to evaluate differences.

Von Frey assay and autotomy

For the von Frey assay and autotomy, pairwise 2×2 Fisher's exact tests were conducted to make comparisons because of the categorical nature of the data and non-normal distributions. Fisher's exact test suits contingency tables with sampling zeros and numerically small entries,³³ which was the case in this study. For autotomy, both frequency and severity of autotomy between groups were compared. To compare severity, scores were split at each point along the autotomy scale in order to reveal any possible significant differences between groups.

Results

Skin temperature

As a control measure, a two-tailed *t*-test revealed no significant difference between skin temperatures of injured and intact paws in Sham group rats [$t(6) = 0.156$, $P = 0.881$]. Mean percentage differences in paw temperatures for Wistar-Schwann, Wistar-DRG, NG, and Sham were 8.45, 6.28, 5.22, and 0.43%, respectively. A one-way ANOVA with Tukey's HSD *post hoc* assessments comparing all groups detected a non-significant difference between groups [$F(3,18) = 2.6261$, $P = 0.082$]. However, the Wistar-Schwann and Sham comparison was close to significant ($P = 0.056$) (Fig. 3A).

Hot plate test

Interestingly, all rats in the Sham group displayed longer withdraw times in the intact paw (1.62 seconds) versus the injured paw (1.45 seconds). A one-tailed test indicated that the sham-operated control surgery significantly decreased the latency of the injured paws ($P = 0.040$). Unlike the Sham rats, all rats in the Wistar-Schwann, Wistar-DRG, and NG groups displayed higher latency in the injured paw versus the intact paw. Mean percentage differences in latency of the injured paw against latency of the intact paw for Wistar-Schwann, Wistar-DRG, NG, and Sham were 104, 132, 183, and 10%, respectively. A one-way ANOVA with Tukey's HSD *post hoc* assessments comparing all groups yielded significant results [$F(3,18) = 5.3957$, $P = 0.008$], with only a significant difference between Sham and NG ($P = 0.005$) (Fig. 3B).

Extensor postural thrust

The mean percentage differences in the EPT of injured legs against intact legs for Wistar-Schwann, Wistar-DRG, NG, and Sham groups were 77.8, 75.9, 75.2, and 6.9%, respectively. A one-way ANOVA with Tukey's HSD *post hoc* assessments comparing all groups was significant [$F(3,18) = 144.58$, $P < 0.0001$], with significant differences between Sham and each repair group ($P < 0.0001$) (Fig. 3C).

Von Frey assay

Across all groups, intact legs gave 100% positive responses. All injured legs in the Sham and Wistar-DRG groups also gave 100% positive responses. However, only 33% of injured legs in the Wistar-Schwann group and 43% in the NG group had 100% positive responses. Wistar-DRG and NG had respective mean positive responses of 81.7 and 82.9%. Pairwise 2×2 Fisher's exact test comparing all groups revealed a significant effect of treatment on scores between Wistar-Schwann and Wistar-DRG ($P = 0.048$), and Wistar-Schwann and Sham ($P = 0.048$), with a difference approaching significance in Wistar-DRG and NG ($P = 0.081$) (Fig. 3D).

Autotomy

Autotomy scores ranged from 0 to 7. In the NG group, one rat was unintentionally discarded before scoring; all other rats were scored and photographed (Fig. 4). No Sham animals displayed autotomy. In the Wistar-Schwann group, 67% of rats self-mutilated toes on the injured leg, as did 60% of Wistar-DRG rats. The NG group had the highest frequency of autotomy with 100% of rats self-mutilating toes on the injured leg, and three rats scoring 7, which was the highest score among all rats. The median scores for Wistar-Schwann, Wistar-DRG, and NG were 3, 3, and 6.5, respectively. Pairwise 2×2 Fisher's exact tests comparing all groups found the effect of treatment on the frequency of autotomy to be significant in comparing NG and Sham ($P = 0.005$), and close to significant in comparing Wistar-Schwann and Sham ($P = 0.076$) and Wistar-DRG and NG ($P = 0.061$). When autotomy scores were divided by severity between scores less than 4, and scores of 4 and higher, pairwise 2×2 Fisher's exact tests comparing repair groups also revealed a difference between Wistar-DRG and NG that was close to significant ($P = 0.061$).

Discussion

Analysis of results

In our prior study (Liu *et al.*²¹), an analysis of morphology and immunogenicity found Wistar-DRG-repaired nerves to have the best myelination and lowest unwanted immunogenicity among repair groups. Wistar-DRG-repaired nerves best matched Sham group nerves in structure and myelin distribution at the proximal stump. At the distal stump, many axons were only partially myelinated, but appeared healthier than the distal stump axons in the other groups. Wistar-Schwann-repaired nerves were similarly myelinated, but with fewer and larger diameter myelin rings. Wistar-Schwann nerves also had fewer and less distinct axons in both proximal and distal stumps compared to the other three groups, suggesting the most pronounced Wallerian degeneration. However, of repair groups, the distal stumps of Wistar-Schwann nerves displayed the axon wave pattern most similar to the Sham nerves.²¹

Those observations largely support the data in this study. The skin temperature test did not distinguish any notable differences in the quality of reinnervation in the repair groups; and the EPT motor evaluation showed that there was no significant effect of treatment on the recovery of function in efferent neurons. However, in the von Frey assay, Wistar-DRG-repaired nerves demonstrated the best recovery. This corroborates the previous histological

analysis of Wistar-DRG having myelination that best matched Sham myelination. It is believed that the light-touch sensation tested by the von Frey assay is primarily mediated by Abeta-fibers, a group of myelinated nerve fibers that innervate mechanoreceptors.^{34,35} In the von Frey assay, the Wistar-DRG performance was better than that of both the NG and Wistar-Schwann-repaired nerves. Wistar-Schwann had the worst performance of the repair groups, and was also significantly worse than the Sham whereas the other groups were not. This lends support to our prior finding that Wistar-Schwann myelination was worse than the other repair groups.²¹ However, for the hot plate test, Wistar-Schwann-repaired nerves' average latency was lower than that of both Wistar-DRG and NG. Temperature receptors are primarily mediated by Adelta- and C-fibers. Adelta-fibers are thinly myelinated compared to Abeta-fibers, and C-fibers are unmyelinated.^{35,36} So, for these fiber groups, it is possible that allotransplanted Schwann cells enhanced the morphology of these nerves, where effective conduction was not dependent on myelination. For autotomy, the frequency and severity of autotomy in Wistar-Schwann and Wistar-DRG were notably lower than in the NG group. The results suggest that both seeded Schwann cells and seeded DRG cells are beneficial in resolving neuropathic pain. Lastly, the Sham-rat results in the hot plate test suggest that incision surgery induces hyperalgesia. A rat study by Znaor *et al.* has shown that while the effect of an exact injury on pain is unpredictable, those rats with hyperalgesia following injury frequently have associated neuroinflammation.³⁷ However, that may not be the case in this study. Liu *et al.*²¹ reported significantly higher serum IFN-gamma and macrophage concentrations in Wistar-Schwann.²¹ But here, the level of autotomy in Wistar-Schwann was better than that in the NG group. The pathways for neuropathic pain are very complex and likely involve significant changes in cortical plasticity³⁸ in addition to neuroinflammatory changes.

Challenges in measuring PNI recovery in a rat model

In addition to the results discussed, this study revealed difficulties in evaluating PNI recovery in rats that are worth discussing. Ideally, multiple measures would have been used for each aspect of recovery tested, especially with the confirmation that the treatments had different effects on different nerve groups. Here, there were two measures used to examine the return of sensory function, but motor function and pain each had only one measure. This study had initially incorporated and carried out two additional measures to evaluate motor recovery: the sciatic functional index (SFI), and the Basso, Beattie, and Bresnahan (BBB) locomotor rating scale.³⁹ The SFI is a gait analysis that uses the inked footprints left by rats on a walking track to measure the relationships between toes and paws. The score is determined by print length, toe spread between first and fifth digits, and toe spread between second and fourth toes. It is an established method used to evaluate motor recovery quantitatively.^{16,22,40} However, the majority of the injured rats displayed autotomy, which was often severe; so it was impossible to accurately calculate the SFI score.

The BBB scale is described as a robust measure of motor function that considers hind limb joint movement, support of weight, level of plantar stepping, and coordination of gait, in a 0–21 scale.⁴¹ It is recommended as an additional test in evaluating PNI recovery, and may be more sensitive than SFI in certain cases.⁴² The straight alley version of the BBB scale has been shown to produce more reliable scores.⁴³ In our preliminary experiments of this study,

BBB testing was done in conjunction with the SFI testing, and the rats ran along a papered straight platform with an open box and bait as reward at the opposing end (data not published). The runs were videotaped, and three investigators blind to the treatments independently reviewed in footage. However, reported scores did not corroborate. The BBB scale uses multiple descriptors to describe each score along the scale, rather than taking the sum of categorized descriptors as in the Glasgow coma scale. Certain descriptors such as consistent plantar stepping and toe clearance merit higher scores, whereas others such as occasional weight-supported plantar steps and occasional front/hind limb coordination merit lower scores. In this injury model, the investigators in this study found that high-end descriptors were often observed concurrently with low-end descriptors to produce perceived scores that differed by a number of points.

Of further interest is also how hyperalgesia and chronic neuropathic pain might confound various measures. The hyperalgesic effect of incision surgery on hot plate latency observed in the Sham rats was overshadowed by functional deficiencies in the repair-group rats, but it is interesting to consider how the other tests might be affected by sensitivity to pain. For example, pain might negatively compromise the potential thrust of the leg during EPT testing, masking the actual extent of recovery.

While previous studies have demonstrated that Schwann cell- and DRG-seeded conduits enhance regeneration, the effect of the seeded cells on specific nerve fiber groups has not been investigated. Together, the results presented in this study and in our prior study Liu *et al.*²¹ begin to differentiate the differences, and seem to suggest that discriminating between nerve types in researching the mechanisms of nerve regeneration is especially worthwhile in the development of new treatment methods.

Conclusion

In current study of complete sciatic nerve transection and repair, the group using allotransplanted DRG neurons achieved the best recovery, significantly outperforming both the group using allotransplanted Schwann cells and the group using nerve guide conduit alone in the Von Frey assay of touch response. Additionally, allotransplanted DRG neuron and Schwann cell-seeded repairs showed lower frequency and severity in an autotomy measure of the self-mutilation of the injured leg due to neuralgia. Our data suggest that in complete nerve transection, surgical repair using seeding nerve guide conduits with allotransplanted DRG neurons and Schwann cells may improve functional recovery, especially DRG neurons, which elicit less of an immune response.

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Figure 1.
Artist's illustration demonstrating the extensor postural thrust (EPT) method.

Figure 2.
Artist's illustration demonstrating the Von Frey assay method used.

Figure 3. Percentage differences between intact and injured legs in (A) skin temperature, (B) hot plate latency differences, and (C) extensor postural thrust (EPT). (D) Percentage of animals with 100% positive responses.

Figure 4.

Autotomy in injured-side paws with scores. Top panel: the group repaired by nerve guide seeded with allotransplanted Schwann cells. Center panel: the group repaired by nerve guide seeded with allotransplanted DRG neurons. Bottom panel: the group repaired by nerve guide alone.