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**Sciatic nerve remyelination and nodal formation following
olfactory ensheathing cell transplantation**

A Thesis Submitted to the
Yale University School of Medicine
in Partial Fulfillment of the Requirements for the
Degree of Doctor of Medicine

by

Mary A. Dombrowski

2007

ABSTRACT

SCIATIC NERVE REMYELINATION AND NODAL FORMATION FOLLOWING OLFACTORY ENSHEATHING CELL TRANSPLANTATION. Mary A. Dombrowski, Masanori Sasaki, Karen L. Lankford, Hajime Tokuno, Christine Radtke, Jeffery D. Kocsis. Department of Neurology and Center for Neuroscience and Regeneration Research, Yale University School of Medicine, New Haven, CT, Rehabilitation Research Center, Veterans Affairs Connecticut Healthcare System, West Haven, CT.

Transplantation of olfactory ensheathing cells (OECs) into injured spinal cord results in improved functional outcome through axonal regeneration, remyelination, and neuroprotection. However, because little is known of the fate of OECs transplanted into injured peripheral nerve, their myelin forming potential requires investigation. To study these issues OECs were isolated from the olfactory bulbs of adult green fluorescent protein (GFP)-expressing transgenic rats and transplanted into a sciatic nerve crush lesions. Five weeks to six months after transplantation the nerves were studied histologically and it was determined that GFP-expressing OECs survived in the lesion and distributed longitudinally across the lesion zone. Immunostaining revealed a high density of isoform Na_v1.6 at the newly formed nodes of Ranvier which were flanked by paranodal Caspr staining. Immunoelectron microscopy for GFP revealed transplanted OECs form peripheral type myelin. These results indicate that transplanted OECs extensively integrate into transected peripheral nerve, form myelin on regenerated peripheral nerve fibers, and reconstruct nodes of Ranvier with proper sodium channel structure.

PUBLICATIONS

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INTRODUCTION

Olfactory ensheathing cells (OECs) are specialized glial cells that guide the regeneration of nonmyelinated olfactory axons from the peripheral nasal epithelium, through the cribriform plate of the ethmoid bone, and into the olfactory bulb. They are located in the olfactory epithelium, olfactory nerve, and the first two outer layers of the olfactory bulb known as the olfactory nerve layer (1). The olfactory neuroepithelium undergoes continuous turnover and sends new axons into the olfactory bulb where they synapse with two cell types responsible for the output signals: mitral cells and tufted cells. Mitral cells are a specific type of neuron that have a primary dendrite, as well as multiple branching dendrites. Tufted cells are similar in structure to mitral cells; however, they have distinct projections into the olfactory cortex. These cells also have distinct molecular phenotypes (2).

Olfactory axons continuously regenerate throughout the lifetime of an organism, and they begin in the nasal epithelium as nonmyelinated, individual axons that form bundles. These bundles, fila olfactoria, migrate from the periphery, cross the pia mater, and then enter the central nervous system (CNS) where they establish connections with neurons in the olfactory bulb (1). More specifically, OECs accompany axons through the lamina propria in the peripheral nervous system (PNS) and into the CNS via the cribriform plate. It is the OECs and their associated axons that surround the outside of the olfactory bulb and create the olfactory nerve layer (3). The olfactory nerve layer is divided into two distinct layers before the olfactory axons continue, unaccompanied by the OECs, into the deeper layers of the olfactory bulb. The two layers are termed the “outer” and “inner”

layer and differ in their molecular expression. Thus, it is primarily the olfactory nerve layer that is rich in OECs and is the target for OEC collection.

OECs surround olfactory axons throughout their segments in the PNS and in the olfactory bulb. Specifically, they act to group the separate receptor axons into fascicles by creating cytoplasmic projections that encase the bundles (3). Also, because the OECs surround the olfactory axons, they act to prevent the axons from coming into contact with other types of glial cells. This is a major reason why OECs are thought to play a critical role in guiding the regrowth of olfactory neurons (4).

The developmental origin of the olfactory system gives clues about the characteristics and function of OECs. Within the olfactory nerve and olfactory bulb there exist cells that originate from the cerebral vesicle, as well as cells that develop from the olfactory placodes. This difference is important because it identifies the cell types as being of central or peripheral origin. It has been demonstrated that astrocytes, neurons, and oligodendroglia develop from the cerebral vesicle, while olfactory neurons and OECs are derived from the olfactory placodes (1). In fact, OECs were originally known as olfactory nerve Schwann cells (SCs). Thus, the peripheral nature of OECs is well established and their bridging function between the PNS and the CNS provokes questions regarding the true potential of these cells.

However, the true characteristics of OECs are not limited to SC properties. Studies of OECs within the CNS demonstrate that they also have properties of astrocytes. For example, Devon et al demonstrated that OECs and astrocytes share the same anatomical location in the olfactory bulb, express glial fibrillary acidic protein (GFAP), and form the bulb's glia limitans (4). Because of the inability to categorize the OECs

based on astrocyte or SC characteristics alone, OECs were designated as their own glial cell type. Furthermore, they are structurally distinct from other glial cells types with an electron rich cytoplasm, intermediate filaments, and irregular nuclei (3).

OECs have multiple markers that allow for their identification, as pertaining to OEC extraction and purification. The outer and inner layers of the olfactory nerve layer are distinguished by their reactivity with p75 and embryonic neural cell adhesion molecule (E-NCAM). The OECs in the outer layer do demonstrate reactivity to the p75 neurotrophin receptor and E-NCAM, while the OECs in the inner layer do not. More generally, OECs in both layers have reactivity to S100 and green fibrillary acidic protein (GFAP). S100 is a calcium binding protein that is associated with intracellular processes such as protein phosphorylation and differentiation and is considered to be a marker for SCs, astrocytes, and oligodendrocytes. GFAP is an intermediate size cytoskeletal protein and is a marker for astrocytes (3). OECs also express multiple trophic factors, transcription factors, and extracellular matrix molecules (5).

Because the OECs are a specialized glial cell that have characteristics of astrocytes, SCs, and oligodendrocytes there has been extensive research regarding the potential of OECs to promote nerve regeneration when transplanted into the CNS. Several studies have demonstrated functional recovery after OEC transplantation in the injured CNS (5, 6, 7, 8, 9). For example, in rats that have undergone complete spinal cord transection, transplantation of OECs results in recovered climbing ability and sensorimotor reflexes (3). While the precise mechanism of this functional recovery is not fully understood, several mechanisms have been suggested including remyelination (4, 5, 10, 11), long axon tract regeneration (7, 12, 13), axonal sparing (8) and plasticity

associated with novel polysynaptic pathways (14, 15). In addition, recruitment of endogenous SCs (16, 17, 18) and remote inhibition of apoptosis of motor cortical neurons (19) have been suggested to contribute to improvement in functional outcome in injured CNS after OEC transplantation. However, it is difficult to compare these studies given the variety of cell preparation and purification techniques, spinal cord injury methods, and OEC markers.

The destruction of myelin in nervous system injury, both central and peripheral, is the target for regeneration research. Peripheral myelin is formed by the plasma membrane of SCs as they wrap around axons and create multiple layers of wrapping mesaxon. Central myelin is different in its formation and structure. In the CNS myelin is also formed by oligodendrocytes, which also wrap their membrane around the axons. When oligodendrocytes form myelin in the CNS, each cell is responsible for myelination of multiple axons. However, in the PNS, SCs only myelinate single axons. This is a potential reason for why demyelinating disease in the CNS is more devastating to neurological function than demyelination and regeneration in the PNS (20). Beyond myelination, the proper anatomical structure of the node of Ranvier, the paranodal region, and the juxtaparanode are essential for proper nerve conduction. Within the node of Ranvier, sodium channels are clustered to allow for saltatory conduction. The isoforms of these sodium channels can give clues as to the maturity of the node. Adjacent to the node is the paranodal region where myelin is in direct contact with the axon. Finally, adjacent to the paranodal region is the juxtaparanode.

There is established data that demonstrates the ability of SCs to remyelinate areas of CNS injury. In CNS injury there is a natural proliferation of SCs that originate in

dorsal roots and surround blood vessels that supply the spinal cord (21). Thus, the natural presence of SCs within areas of CNS injury make them an interesting candidate for nerve repair. What inhibits the benefit of SC transplantation is the presence of astrocytes within many CNS lesions, and these are thought to prevent SCs from migrating into the CNS. Thus, in the presence of astrocytes, SCs do not thrive in CNS lesions. Because OECs share properties of both SCs and astrocytes, and also coexist with astrocytes in their native environment of the olfactory bulb, their potential therapeutic effect in CNS injury is intriguing (10).

A study by Sasaki et al investigated the ability of OECs to form myelin within an area of spinal cord injury. In adult rats, the dorsal funiculus was transected and OECs were transplanted into the lesion site. It was demonstrated that five weeks after transplantation, the OECs survived within the lesion site, aligned longitudinally along the axon fibers, and did form peripheral type myelin. However, there are other studies that have created similar models of OEC transplantation into spinal cord injury and believe that the myelin is formed by SCs that migrate into the lesion site from the periphery (5). The regeneration of peripheral type myelin in areas of spinal cord injury has been demonstrated to occur in the absence of OEC transplantation, thus supporting the theory of SC migration. Therefore, it is difficult to differentiate between myelin that may have been formed by the transplanted OECs and that formed by endogenous SCs.

Recently, it has been shown that after spinal cord remyelination by transplanted OECs derived from green fluorescent protein (GFP) expressing rats, nodes of Ranvier achieve sodium and potassium channel organization similar to that exhibited at mature central nodes. A mature node of Ranvier is constructed such that sodium channels

populate the nodal area and potassium channels are located in the juxtaparanodal regions. Thus, in mature CNS nodes there exists a clustering of $\text{Na}_v1.6$ at remyelinated nodes and $\text{K}_v1.2$ in the juxtaparanodal region (22). Within an immature node the $\text{Na}_v1.2$ isoform predominates. As myelination occurs, $\text{Na}_v1.2$ is replaced by $\text{Na}_v1.6$, indicating node maturity (23). It has been shown previously that engrafted GFP-expressing SCs participate in myelination of regenerated peripheral nerve fibers and that the $\text{Na}_v1.6$ sodium channel is reconstituted on the regenerated axons (24). These results demonstrate that exogenous myelin-forming cells are able to contribute to the establishment and maintenance of mature ion channel distribution on remyelinated axons. Moreover, the remyelinated axons support rapid and secure impulse conduction after demyelination in the CNS.

Although there is extensive, ongoing research on transplantation of OECs into the injured CNS, there are only limited studies regarding the role of transplanted OECs in the PNS. The natural process of axonal degeneration that occurs after injury to the PNS is termed “Wallerian degeneration.” Once peripheral nerve has been injured the distal nerve stump begins to degenerate. Within 24-48 hours there occurs destruction of the myelin sheath, which is followed by fragmentation of the myelin into ellipsoids. The ellipsoids divide such that one exists near the node of Ranvier and one in the internodal region. Subsequently, there is a proliferation of SCs and invasion of macrophages. The Wallerian degeneration that takes place in areas of CNS injury is different because there is no formation of ellipsoids or fragmentation of the internodal region. Also in the CNS, myelin debris is removed by phagocytes and this proceeds at a slower rate than in the PNS (25).

After peripheral nervous system injury, the natural presence of SCs create an environment that allows axonal regeneration to occur. For example, after a peripheral nerve injury, SCs will change their phenotype from a myelin forming cell to one that creates trophic factors, cell adhesion and extracellular matrix molecules, and the framework for axonal regeneration (26). Eventually, axons in the peripheral nerve will sprout and grow towards distal targets and then be remyelinated by SCs from the distal stump (24).

To investigate the myelin forming potential of OECs and their ability to compete with endogenous SCs in the PNS, OECs from adult GFP-expressing transgenic rats were transplanted into a crush lesion site in the sciatic nerve of non-GFP adult rat. The study results demonstrate that GFP-OECs survive and distribute longitudinally along regenerated axons and remyelinated the injured axon after transplantation into injured peripheral nerve. Furthermore, the regenerated fibers show reconstructed nodes of Ranvier with mature sodium channel organization. Thus, transplanted GFP-OECs prepared from adult rat olfactory bulb are able to remyelinate regenerated peripheral nerve fibers and restore proper nodal structure in injured PNS.

HYPOTHESIS AND STATEMENT OF PURPOSE

Previous studies have demonstrated the myelin forming capacity of OECs when transplanted into areas of CNS injury. Thus, we hypothesize that OECs will retain their myelin forming potential when transplanted into a peripheral nerve lesion. We believe that OECs will allow for axonal regeneration and the formation of peripheral type myelin within the lesion site. It is hypothesized that the OECs will migrate beyond the lesion site to induce guided axon regeneration. Also, it is likely that the regenerated nodes of Ranvier will have proper ion channel structure, allowing for restoration of normal axon conduction velocity. The primary purpose of this study is to investigate the myelin forming potential of OECs within the PNS. This will lead to a better understanding of the basic characteristic of OECs and allow there to be confidence in previously published studies that demonstrate myelin formation by transplanted OECs in CNS lesions.

MATERIALS AND METHODS

Contributions to the text for this section were made by Dr. Karen L. Lankford, Dr.

Masanori Sasaki, Dr. Christine Radtke, and Dr. Jeffery Kocsis.

Isolation and Characterization of Donor OECs

Production of all reagents was performed by Dr. Karen L. Lankford.

To obtain freshly isolated OECs, olfactory bulbs were removed from 4 to 8 week-old transgenic rats expressing GFP ["green rat" CZ-004, SD-Tg (Act-EGFP) CZ-004Osb; SLC, Shizuoka, Japan] and the meninges were removed to minimize contamination. After removing the caudal one-third of the bulb, the white matter was extracted and discarded to isolate the outer nerve layer. Tissue was finely minced with a pair of scalpel blades (#10) on plastic culture dishes and incubated for 25 minutes in collagenase A (0.75 mg/ml; Roche, Indianapolis, IN), collagenase D (0.75 mg/ml; Roche), and papain (12 U/ml; Worthington, Lakewood, NJ) in calcium-free complete saline solution (CSS) with a trace of cysteine for 25 minutes at 37°C on a rotary shaker in a CO₂ incubator. The tissue suspension was then centrifuged for 7 minutes at 300 x g.

Following this, the pellet was resuspended in 2 ml of Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) with 10% fetal calf serum (FCS) using gentle mechanical trituration with fire-polished pasture pipettes that had successively reduced diameters. The volume of media was immediately increased to 20 ml. Undissociated pieces of tissue were allowed to settle for 2 minutes before transferring the cell suspension to another culture tube and centrifuging as before. Cells were washed twice, resuspended, and preplated for 1 hour in a culture flask at 37°C in a

CO₂ incubator. Nonadherent cells were gently washed off with DMEM, and the cells were centrifuged and resuspended three times in DMEM. Cells were counted and concentrated to 3.0×10^4 cells/ μ l just before transplantation. P75- and S100-positive cells were counted in short-term cultures, made from cell suspensions used for transplantation, to assess purity of the cells. Over 95% of the cells were positive for p75 and S100.

Crush Lesion and OEC transplantation

Experiments were performed in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. The Veterans Affairs Connecticut Healthcare System Institutional Animal Care and Use Committee approved all animal protocols. Adult wild Sprague Dawley rats (200-225g) were used for this experiment (n=34). The rats were first anesthetized with ketamine, 75 mg/kg, intraperitoneally (i.p.) and xylazine, 10 mg/kg i.p. The sciatic nerves were exposed near the piriformis tendon and crushed for 20 seconds with fine forceps (Dumont #5). This procedure transects all of the axons in the nerve, but the epineurium remains intact, allowing the axons to regenerate into the distal nerve segment. Immediately after the crush injury was performed, 0.5 μ l of a GFP-OEC suspension (3.0×10^4 cells/ μ l), or DMEM for sham control, was injected just distally to the crush site using a glass pipette (40 μ m tip diameter) attached to a 1 μ l Hamilton syringe (24).

Whole mount images of the living nerve

Five weeks after transplantation, animals (n=3) were deeply anesthetized with 50 mg/kg sodium pentobarbital i.p. and decapitated. The sciatic nerve from transplanted rats was extracted and desheathed with fine forceps. Some samples were put in 4% paraformaldehyde for five minutes and teased with fine forceps. The sections were examined by conventional fluorescence microscopy (Nikon Eclipse 800; Spot RT Color CCD camera; Diagnostic Instruments).

Immunohistochemistry.

Immediately after the OECs had been prepared and isolated, they were resuspended in 10 ml of culture media. They were then washed and plated in 8-well poly-L-lysine-coated chamber slides in DMEM with 10% FCS and penicillin–streptomycin. Cells were plated for 3-7 days and then fixed for 10 minutes in methanol that had previously been on ice. The cells were then incubated in primary antibody overnight at 4°C. The antibodies used were rabbit anti-p75 (1:1000; Chemicon, Temecula, CA), mouse monoclonal anti-S100 (1:1000; Chemicon), rabbit anti-cow S100 (1:400; Dako, Carpinteria, CA), and mouse monoclonal anti-GFAP (1:1000, SMI22; Sternberger Monoclonals, Lutherville, MD). Following this, cells were again incubated with Alexa Fluor 488 goat anti-mouse (1:1000; Molecular Probes, Eugene, OR) and Alexa Fluor 594 goat antirabbit (1:1000; Molecular Probes) and placed on a shaker at room temperature. Cells were then counterstained on a shaker for one hour with Hoechst 33358 (1:1000; Molecular Probes). Images of the cells were taken with a Spot camera (Diagnostic Instruments, Sterling Heights, MI) and Spot Advanced software. Finally, the

percent of OECs within the sample was determined using merged images of p75, S100, and GFAP with Hoechst nuclear stain.

(The following immunohistochemistry was performed by Heather Mallozzi.)

Sciatic nerves from the sham control (n=5), and GFP-OEC transplanted rats at 5 weeks (n=20) and 6 months (n=3) following transplantation were processed for immunocytochemistry (27, 28). Rats were deeply anesthetized with ketamine/xylazine and perfused transcardially, first with phosphate buffer saline (PBS) and then with ice-cold 4% paraformaldehyde in 0.14 M Sorensen's phosphate buffer, pH 7.4. Sciatic nerves were excised and placed in fresh fixative to achieve a total fixation time of 25 minutes. Tissue was rinsed several times with PBS and cryoprotected in 30% sucrose in PBS overnight at 4°C. Ten micrometer cryosections of the sciatic nerve were cut and mounted on glass (Fisher Superfrost Plus) slides. The sections were processed for double immunofluorescent staining for the detection of Na_v1.2, Na_v1.6, and Caspr or with single immunolabeling for Neurofilament (NF). The primary antibodies used were as follows: polyclonal Na_v1.2 (1:100; Alomone Labs, Jerusalem, Israel), polyclonal Na_v1.6 (1:100; Alomone Labs), monoclonal NF (1:1000; Sigma) and monoclonal Caspr (1:300) (29). Secondary antibodies used were as follows: goat anti-rabbit IgG-Cy3 (1:2000; Amersham Biosciences, Piscataway, NJ) and goat anti-mouse IgG-Alexa Fluor 633 or Alexa Fluor 546 (1:1000; Invitrogen, Eugene, OR). The sections were examined by confocal microscopy (Nikon Eclipse E600 microscope; Simple PCI; Compix Imaging Systems, Cranberry Township, PA) and with conventional fluorescence microscopy (Nikon Eclipse 800; Spot RT Color CCD camera; Diagnostic Instruments).

Immunoelectron microscopy

Seven weeks following transplantation, animals (n=3) were deeply anesthetized with 50 mg/kg sodium pentobarbital given i.p. and perfused transcardially with PBS. This was followed by 4% paraformaldehyde/0.02% glutaraldehyde in PBS. Sciatic nerves were excised, postfixed overnight in 4% paraformaldehyde, and embedded in 3% agar for vibratome sectioning. Free-floating sections that were 150 μ m thick were incubated in 2% normal goat serum for 30 minutes and then in rabbit anti-GFP antibody (1:2000; Chemicon) overnight at 4°C. These sections were then incubated overnight with an anti-rabbit biotinylated secondary antibody (Sigma, St. Louis, MO) and then incubated for 1 hour using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) following standard protocols. The sections were postfixed with 1% osmium tetroxide for 4 hours, dehydrated in graded ethanol solutions and embedded in Epox-812 (Ernest Fullam, Latham, NY). Ultrathin sections were cut as described above but were not counterstained.

Electrophysiology

Electrophysiological studies were performed by Dr. Hajime Tokuno.

Sections of host OEC transplanted sciatic nerve (1.5-3.0 cm) were removed and electrophysiology studies were performed. The nerves were desheathed and placed in a nerve recording chamber. The nerve recording chamber consists of 2 pairs of Ag-AgCl electrodes that were separated by 1 cm. The nerve segments were placed across the

electrodes and the proximal nerve ending was stimulated. At the distal end of the nerve segment the monophasic compound action potential was recorded. Throughout the experiment, the central portion of the nerve segment was perfused with warmed (37°C) Ringer's solution (30).

When the proximal end of the nerve segment was electrically isolated, the peak of the action potential in response to a stimulus was recorded at varying distances. The conduction velocity was determined by dividing the distance traveled by the onset or peak latency. This provided an accurate measurement of conduction velocity in the regenerating nerve segment. The electrophysiology studies were performed at 2, 3, 4, 7, 8, and 10 weeks post OEC transplantation.

RESULTS

GFP-OECs transplanted into crushed sciatic nerve lesion

Whole mount images of live and fixed nerve were prepared at five weeks post OEC transplantation and revealed that GFP-OECs survived in the lesion site and distributed longitudinally along the regenerated axons several millimeters from the injection site (Fig. 2A, 2B). When the sciatic nerve was teased, it revealed that the GFP-OECs were associated with individual axons within the lesion site (Fig. 2C1). Areas of intense fluorescence along the axon were detected and represent the dense cytoplasmic regions and nuclei of the GFP-OECs. There were sections within the areas of concentrated fluorescence where no GFP fluorescence was detected and these areas were representative of putative nodes of Ranvier (Fig. 2C2). Confocal images demonstrated an abundance of GFP-expressing OECs at the lesion site and they were oriented longitudinally along the nerve (Fig. 3A). NF immunostaining images indicated the close association between the regenerated axon fibers and the GFP-expressing OECs (see inset Fig. 3A).

Confocal images taken at six months post transplantation showed the presence of GFP-OECs indicated by intense green fluorescence (Fig. 3C). In contrast to the results 5 weeks following transplantation, the GFP intensity was slightly decreased. This demonstrates the ability of transplanted OECs to survive in the lesion site for at least several months.

Na_v1.6 is located at the newly formed nodes of remyelinated axons

Nodes of Ranvier within the GFP-OEC transplanted sciatic nerves were identified by flanking paranodal Caspr immunofluorescence (Fig. 3B) (22, 31, 32). At 5 weeks after engraftment of OECs nodes were identified by their association with adjacent GFP-OECs and Caspr reactivity. These areas were characterized by GFP fluorescence in the region of the terminal paranodal loops, as well as within the outer layer of the GFP-OEC sheath (Fig. 3B, inset upper right). This confirms the association of the transplanted OECs with the remyelination of host axons. Also, intense Na_v1.6 was observed at the putative nodes of Ranvier (Fig. 3B inset lower left). We observed Na_v1.6 staining at most nodes, whereas detectable Na_v1.2 immunostaining was not apparent at any nodes in the sciatic nerve (Fig. 5). Similar results have been demonstrated in the CNS models of remyelinated lesion by transplanted GFP-OECs (22).

Furthermore, 5 weeks after OEC engraftment in the PNS model, almost all nodes bounded by GFP-OEC myelin sheaths exhibited Na_v1.6 staining (Fig. 5 A-D), while Na_v1.2 immunolabeling was not observed at any nodes (Fig. 5 E-F). The Na_v1.6 labeling was limited to the nodal domain and was not observed in paranodal and juxtaparanodal regions or beneath the myelin sheath in remyelinated axons. This suggests that the transplanted GFP-OECs are able to contribute to the specific clustering of sodium channels at the newly formed nodes of Ranvier.

Immunoelectron microscopy of GFP-OECs forming myelin

Immunoelectron microscopy for GFP-reaction product was performed to establish that the OECs derived from GFP rats were indeed responsible for the remyelination (Fig. 6). The cytoplasm and nuclei of cell profiles surrounding myelinated axons contained intense reaction product and GFP⁺ cells were determined to be in direct contact with host axons. GFP-reaction product was clearly evident in the cytoplasm of most cells that formed the well defined multi-laminate structures characteristic of myelin. In longitudinal sections of myelinated axons, intense reaction product can be seen in the cytoplasm of the myelin-forming cell (Fig. 6A1, 6A2). The myelin formed has characteristics of peripheral type myelin given the large cytoplasmic and nuclear regions (Fig. 6B1). Some unlabeled cells forming peripheral-like myelin could also be detected within transplanted lesions in the same areas as the labeled cells. A limited number of GFP⁺ cells did surround host axons, but did not form myelin.

Electrophysiology of transplanted sciatic nerve

Initial electrophysiological results of the transplanted sciatic nerve as compared to a normal control reveal greater than 50% recovery of nerve conduction velocity (Fig. 8). The compound action potential (CAP) of the sciatic nerve of normal controls was 44.8 m/s. Eight weeks after OEC transplantation, the conduction velocity of the sciatic nerve of the transplanted crushed nerve was 25.6 m/s. Nerve conduction studies on crushed sciatic nerves without OEC transplantation were not performed. We would expect remyelination to occur naturally and for there to be some level of nerve conduction restoration in this case, but not to the same extent as in the transplanted nerves.

When electrophysiology studies were performed at more frequent intervals it was determined that at 2 weeks post GFP-OEC transplantation into the sciatic nerve crush injury there were no CAP observed. However, at 3 weeks post transplant conduction velocity began to be restored. Four weeks post transplant there was a reemergence of CAP signal. By seven weeks post transplant it was observed that the peak latencies were shorter indicating faster conduction velocities, and at ten weeks post transplant there was an obvious increase in conduction velocity (Fig. 9).

DISCUSSION

There is much evidence regarding the potential of OECs to form myelin and to have beneficial effects within the context of CNS injury. In a study by Sasaki et al transplantation of GFP-expressing rat OECs into a dorsal spinal cord transection lesion resulted in functional improvement and remyelination in a set of regenerated axons. The dorsal funiculus of adult, female Sprague Dawley rats was transected and GFP expressing OECs were immediately transplanted into the lesion site via a micropipette injection technique. The benefit of transplanting cells from a GFP transgenic rat is that the green fluorescence in the cytoplasm and nuclei of the transplanted cells makes them easy to identify in the host. In the host rats, the new myelin that was formed contained GFP as confirmed by light microscopy and immunoelectron microscopy. Thus, the transplanted OECs were confirmed to be the parent cell (19).

Arguments regarding the true myelinating potential of OECs revolve around the idea that rather than actually forming new myelin, the transplanted OECs recruit SCs endogenous to the host which are responsible for the myelin formation. The study did in fact identify cells within the lesion zone that were negative for GFP, but this was outweighed by the presence of GFP+ myelin. Also, it is argued that the transplanted OECs could have been contaminated by SCs endogenous to the donor. It is possible that there could be some amount of SC contamination due to the presence of SCs around vessels in the olfactory bulb, but the study states this would not account for the immunohistochemistry confirmed cell culture purity and the degree of myelin formation (5).

Previous work has demonstrated the ability of OECs to form myelin with a peripheral-like pattern when transplanted into a CNS lesion (5, 6, 10, 11, 22, 33, 34). The first in vivo study to demonstrate regrowth of peripheral type myelin in a CNS lesion was performed on injured, adult rat spinal cord. In this study, clonal OECs were transplanted into an area of rat spinal cord that had been injured by X-irradiation and then injected with ethidium bromide. The study demonstrated that the cells formed peripheral type myelin within the lesion site as indicated by the presence of P0 glycoprotein within the myelin sheaths (10). In another study, this same method of spinal cord injury was performed and neonatal rat OECs were injected into the lesion site six days post injury. They studied the transplanted axons histologically and found that there were remyelinated axons near the lesion site and beyond. Furthermore, to investigate the functional recovery electrophysiological studies were performed and revealed improved conduction velocity, as well as a greater distance of action potential conduction. Therefore, this supports the potential of OECs to remyelinate areas of spinal cord injury with resulting functional recovery (11).

Limited research has been done on the regenerating and myelinating potential of OECs when transplanted into a PNS lesion. It is well known that following peripheral nerve transection, axons in the proximal nerve region sprout and can regenerate toward peripheral targets, often leading to target reconnection (35). Endogenous SCs, derived from the distal nerve segment, subsequently remyelinate these regenerated axons leading to rapid impulse conduction. However, because OECs derived from GFP expressing rats were used, we could distinguish between transplanted and endogenous cells that contributed to the remyelination. In one study the sciatic nerve of adult rats were

transected and the stumps were bridged with silicone tubing. When OECs were added to the laminin gel filling the tubing it was found that axonal regeneration occurred in 50% of the rats. Thus, it was determined that OECs promote nerve regeneration in severe peripheral nerve injury models (36). Because the data on OEC transplantation into PNS injury are limited, it is important that this model be investigated regarding the myelin forming potential of the cells.

In this study, we demonstrate that when transplanted into a crush injury of the sciatic nerve of a rat host, GFP-expressing OECs survive and distribute longitudinally along regenerated axons. NF staining reveals the regenerated axons to be surrounded by GFP fluorescence in the nuclei and dense cytoplasmic areas of the OECs. Proper ion channel structure at the nodes of Ranvier in the regenerated axons was also evaluated. In order to evaluate the distribution of ion channels in the regenerated axon, it is important to understand basic myelinated axon physiology.

All myelinated axons are divided into distinct regions which include the node of Ranvier, the paranodal region, the juxtaparanodal region, and the internode. Each region has a concentration of specific ion channel types and proteins. The node of Ranvier consists of $\sim 1\text{-}\mu\text{m}$ spaces in the myelin sheath where there exists a concentration of voltage gated sodium channel that allow for forward conduction through depolarization of the membrane (37). Ten sodium channel isoforms have been identified in mammals and $\text{Na}_v1.6$ is the dominant sodium channel at mature nodes (38). The function of each sodium channel isoform has not yet been identified. $\text{Na}_v1.1$, $\text{Na}_v1.2$, $\text{Na}_v1.3$, and $\text{Na}_v1.6$ have been found to be expressed in the CNS, and $\text{Na}_v1.6$ is found in nodes of Ranvier in both the CNS and PNS. Thus, because this study demonstrated immunostaining of

Na_v1.6 and lack of Na_v1.2 in the transplanted hosts, it can be determined that mature nodes of Ranvier with proper sodium channel structure were formed.

The paranodal region flanks the node of Ranvier and consist of cytoplasmic wrappings around the axon. The paranodal junction, defined as the region between the node of Ranvier and the paranode, is characterized by the protein Caspr/Paranodin. This region is multifunctional in that it allows myelin to be attached to the axon, as well as prevents both lateral and periaxonal diffusion (37).

The juxtaparanodal region is defined as the area of axon that is beneath the end of the myelin sheath and is located just beyond the paranodal region. This area is heavily populated by potassium channels, and their subtypes include K_v1.1, K_v1.2, K_v1.4 and K_vβ2. The potassium channels are responsible for the repolarization of the axon, firing frequency, and neurotransmitter release (37,39). Potassium channels are constructed of four α-subunits and 0-4 β-subunits. There is a difference during development of the CNS and PNS regarding the location of potassium channels. In the CNS the potassium channels are located in the juxtaparanodal region, whereas in the PNS, they begin at the node of Ranvier and then migrate out into the paranodal and juxtaparanodal regions (22).

In the context of OEC transplantation into areas of CNS and PNS injury, it is important to evaluate whether or not the regenerated axon has proper ion channel structure to allow for restoration of normal conduction velocity. In a study by Sasaki et al, it was demonstrated that when OECs were transplanted into an area of CNS injury that the nodes of Ranvier had correct sodium and potassium channel structure. The new node was characterized as mature by the presence of Na_v1.6 and the juxtaparanodal region

contained K_v1.2. Most importantly this study revealed functional recovery as demonstrated by near normal conduction velocities at 3 weeks post transplant (22).

This study also showed clear evidence of GFP immuno-reactivity as seen on an ultrastructural level in the cytoplasm of cells within the lesion site that were forming peripheral-like myelin. Myelin profiles, observed on an electron micrograph level, revealed a relatively high proportion of 3,3'-diaminodenzidine (DAB) positive cells associated with myelinated axon profiles. Over half of the myelinated profiles were associated with GFP+ cells, providing similar results to that of the dorsal transection model in rat spinal cord (5). Both endogenous SCs and transplanted OECs contribute to myelination of the regenerated axons. Taken together, it is concluded that within an area of peripheral nervous system injury, transplanted OECs can compete with endogenous SC and form new myelin on regenerated axons.

An important consideration in interpreting the results of the present study is the possibility that SC contamination in the OEC preparation might contribute significantly to the population of surviving cells in OEC transplanted lesions. As pointed out by Plant et al and further discussed by Boyd et al, SCs from nerves innervating blood vessels of the olfactory bulb may be a source of SC contamination in OEC preparations (8, 21). Although p75 and S100 staining can distinguish between OECs and other cell types within the olfactory bulb, they cannot distinguish between OECs and SCs. While we cannot rule out the possibility of minor SC contamination in our OEC cultures, the comparable degree of SC and OEC integration into the transplanted nerve is incompatible with minor SC contamination in the OEC cultures (24). Moreover, the extent of remyelination by equal numbers of transplanted genetically marked SCs and OECs was

similar in a spinal cord demyelination model. This suggests that a small contaminant of SCs in the OEC preparation did not account for the remyelination by the transplanted OECs (40).

Importantly, in a study by Franklin et al an immortalized OEC line was used for *in vivo* remyelination. This further supports the idea that OECs are able to form myelin under appropriate conditions. It was found that the OECs myelinated the axons in a manner similar to that of SCs. The importance of using a clonal cell line for the OEC transplant was to ensure that the culture was not contaminated with phenotypically different cells (10). However, it should be emphasized that the preparation of p75⁺ OECs from the adult olfactory bulb in the current study is diverse in culture and includes cells with flattened and spindled shapes that may have different fates *in vivo*.

To further investigate the level of nerve repair after OEC transplantation, electrophysiology studies were performed. Electrophysiology studies measured the conduction velocity of transplanted nerves, as compared to normal controls. By electrically isolating one end of the nerve segment and then providing a stimulus, the amount of time required for the action potential to reach specific distances from the stimulus was recorded. When analyzing the data, it is important to analyze the onset or peak latency. Onset latency is defined as the time between the stimulus artifact and the beginning of the positive slope in the upswing of the plotted action potential. The peak latency is defined as the time from the stimulus artifact to the highest point, the peak, where the slope of is zero. To determine the conduction velocity the distance traveled is divided by the peak or onset latency. In injured nerve, the conduction velocity is dramatically decreased, and as reinnervation and remyelination occur, the conduction

velocity is restored. In our study it was determined that at 8 weeks post OEC transplant, the conduction velocity of the sciatic nerve segment studied was 25.6 m/s. This is compared to the conduction velocity of the normal host sciatic nerve which was determined to be 44.8 m/s.

The electrophysiology studies were subsequently repeated at more frequent intervals post OEC transplantation. It was only at 3 weeks post transplantation that recovery of conduction velocity could be appreciated and at 4 weeks a reemergence of the CAP was seen. Over time shortening of the peak latencies, and this increased conduction velocity, continued to be observed. Further electrophysiology studies could include data on the conduction velocity of sciatic nerve with a crush injury and no transplantation of OECs. This would provide a good comparison of the conduction velocity recovery in natural peripheral nerve remyelination to the faster recovery after OEC transplantation.

These results indicate that transplanted OECs derived from adult rat olfactory bulb, myelinate regenerated peripheral nerve fibers and appropriate nodal sodium channels are formed on the remyelinated axons. The extent of remyelination was similar to that reported for SC transplantation into the same model system suggesting that this unique population of cells within the olfactory bulb is capable of forming peripheral-like myelin (24). While nerve regeneration is very successful in the nerve crush injury model without cell transplantation, following full transection with nerve reattachment functional outcome is more limited. Given that transplantation of peripheral myelin-forming cells can integrate into peripheral nerve and contribute to repair, it will be important to

compare in the future the relative efficacy of SC and OEC transplants in nerve repair following engraftment into fully transected peripheral nerve.

One concern in regards to OEC transplantation is the possibility that immunosuppression would be required for OEC survival. In our study the host rats were not immunosuppressed after the OEC transplant because the transplanted cells were from the same strain of Sprague-Dawley rat. Although there is always a concern for rejection in allografts, especially when the transplanted cells contained a GFP transgene, rejection of the transplanted OECs was not encountered.

Future research that will aid in the understanding of the potential of OECs to form myelin lies in the area of OEC identification. Currently, OECs are identified by their expression of p75, S100, and GFAP. However, these markers are also expressed by SCs, which makes it difficult to definitely assess the purity of OEC cultures. It is also argued that without a marker specific to OECs, it cannot be concluded that OECs transform into SC like cells that form peripheral type myelin when transplanted into an area of CNS injury. Because the natural attempt at repair of CNS injury involves migration of SCs into the lesion, the host's SCs and the transplanted OECs cannot be differentiated (41).

A new controversial marker, calponin, has recently been proposed to distinguish phenotypically OECs and SCs. Calponin is an actin binding protein that plays a role in smooth muscle contraction and it is not found in SCs (42). In a study by Rizek et al, it was determined that fetal rat bulbar OECs express calponin and SCs from adult rat sciatic nerve do not (41). However, there are some studies that do not support this conclusion. In a study by Ibanez et al the expression of calponin by adult OECs was examined both in

vivo and in vitro. It was determined that calponin was associated with fibronectin positive fibroblasts in the olfactory mucosa and olfactory bulb meningeal cells. However, it was not associated with expression of S100 or neuropeptide Y positive OECs. In vitro, the fibronectin expressing fibroblasts and meningeal cell expression was the same. Calponin was not associated with p75 and S100 expressing OECs in vitro (43). Determining the contribution of calponin to the identification of OECs would be a crucial next step in experimentation and could allow further identification of transplanted OECs in PNS injury models. If calponin proves to be a marker for OECs, the purity of OEC cultures used for transplantation could be assessed. Future studies would be able to determine with certainty that OECs are solely responsible for the formation of peripheral type myelin when transplanted into an area of peripheral nerve injury.

Autologous transplantation of OECs into humans with spinal cord injury is the next step in applications of this research. Currently, multiple studies have been performed assessing the safety and efficacy of autologous OEC transplant. Questions exist regarding the best source of OECs for transplantation, how well the cells will survive in the lesion site, if they will migrate beyond the lesion site, and if there will be any functional improvement in patients over time. A study by Feron et al investigated the safety of OEC transplant in humans with spinal cord injury at the thoracic level. This was a single, blind Phase I clinical trial and the hosts were three men, aged 18-55. The participants were rigorously screened for psychosocial stability, with the hope that they would be amenable to study follow up for at least three years. OECs used for transplant were retrieved by biopsy of the patients' nasal septum near the cribriform plate and then purified and cultured in vitro. This study reported that up to one year post transplant, the

patients reported having no neuropathic pain and were assessed to have no change in neurological or psychosocial status. Furthermore, MRI data at one year post transplant indicated no overgrowth of transplanted cells and no evidence of syringomyelia (44). This study supports the idea that autologous OEC transplantation in humans is feasible and the current data supports its safety.

Questions exist regarding the survival of human OECs when transplanted into areas of spinal cord injury. Deng et al investigated this issue by separately transplanting OECs from both rats and humans into the spinal cord of athymic rats. The immunodeficiency of the rats allowed for analysis of human OECs within the rat model. The study indicated that after transplantation, both human and rat OECs quickly stopped dividing and showed similar abilities to survive and migrate in the host. This study gives confidence to the use of OECs in human spinal cord injury (45). Also, because OECs can be obtained from the human nasal epithelium, simple biopsy could be used to obtain cells for autologous transplantation, thus eliminating the need for immunosuppression.

Another method of obtaining human OECs for transplantation is from human cadaver donors. While obtaining OECs from nasal mucosa biopsy for autologous transplants is under investigation, the recovery of OECs from the olfactory bulbs and olfactory mucosa of adult, cadaver donors is another cell source. A study by Miedzybrodzki et al determined that OEC culture after up to 20 minutes of warm ischemia time was successful (n=10). Secondly, in seven cases, OECs were retrieved from cadaver olfactory mucosa and this was determined to be a more reliable source of cells (46). Thus, although immunosuppression would be an issue for cadaver OEC transplantation, this source remains a viable option.

There is controversy in the literature regarding reports of successful OEC transplantation in humans. One researcher has been particularly aggressive in transplantation in humans and has performed greater than 400 OEC transplants in patients with spinal cord injury. The OECs used for transplant were derived from aborted fetuses. Dobkin et al studied seven of these patients with chronic spinal cord injury to assess for functional recovery and safety of this experiment. It is reported that the method is not safe or efficacious. Furthermore, of the seven patients studied, five were reported to suffer complications such as meningitis, and none of the patients studied had any clinically significant functional recovery (47). Therefore, this study indicates that while the data on OEC transplantation is vast and expanding, there are more questions to be answered before OEC transplantation in humans can become standard therapy.

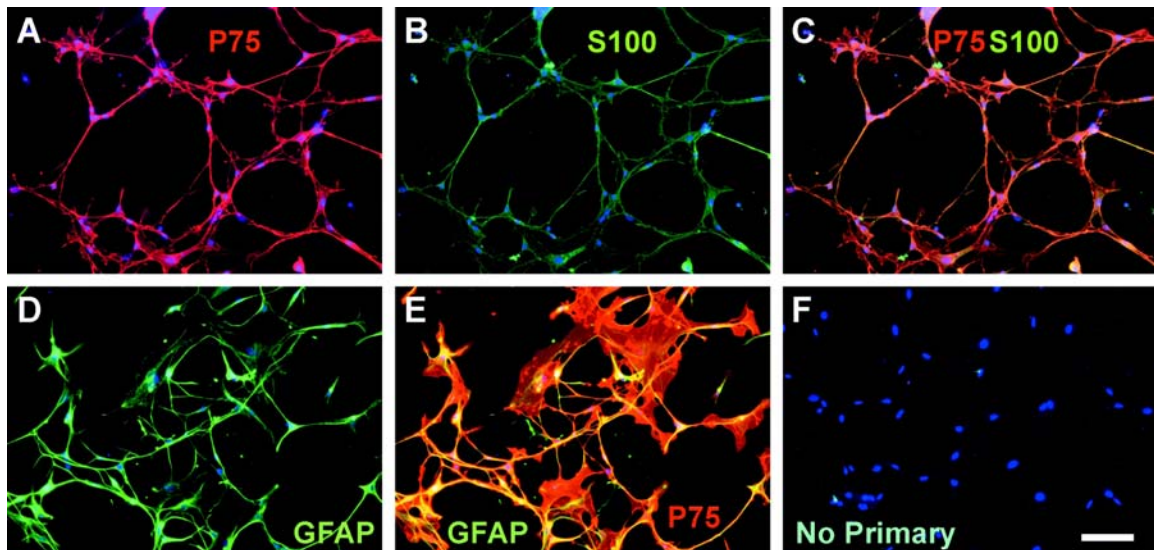
Results of the current study reveal that at five weeks post transplantation the OECs survived within the lesion site, oriented longitudinally along the transection zone, and that peripheral type myelin expressing GFP was present. Furthermore, regenerated nodes of Ranvier demonstrated mature structure with proper sodium channel distribution. Nerve conduction studies revealed restoration of conduction velocity, and this has great implications for functional recovery of the hosts. This data allows for there to be confidence in the current data on OEC transplantation into CNS injury, since the properties of OECs have been elucidated and their value to the field of nerve regeneration has been confirmed.

FIGURES

Figure 1

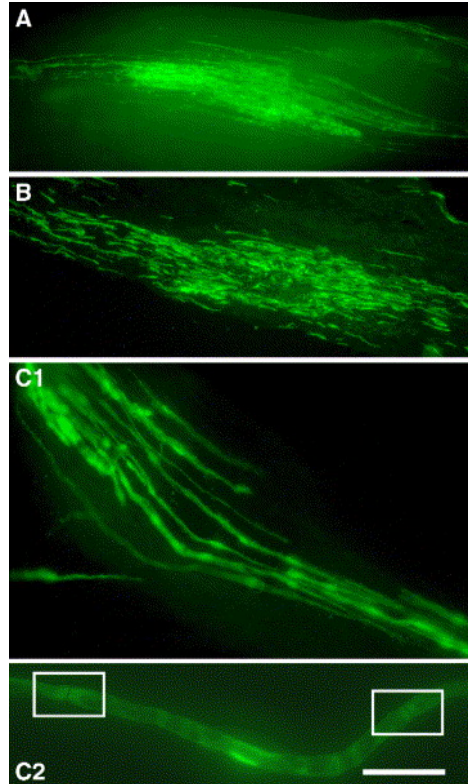
Images produced by Dr. Karen L. Lankford.

The same imaging was performed by Mary A. Dombrowski during experimentation.

**Olfactory ensheathing cells in culture with Hoechst nuclear staining**

The OECs were stained for p75 (A), S100 (B), and GFAP (D). Merged images of S100 (C) and GFAP (D) immunostaining with p75 indicated extensive co-localization. Greater than 95% of cells were p75 and S100 positive. (F) represents a control section with nuclear staining and only secondary antibody staining. Scale bar: A-F = 100 μ m.

Figure 2



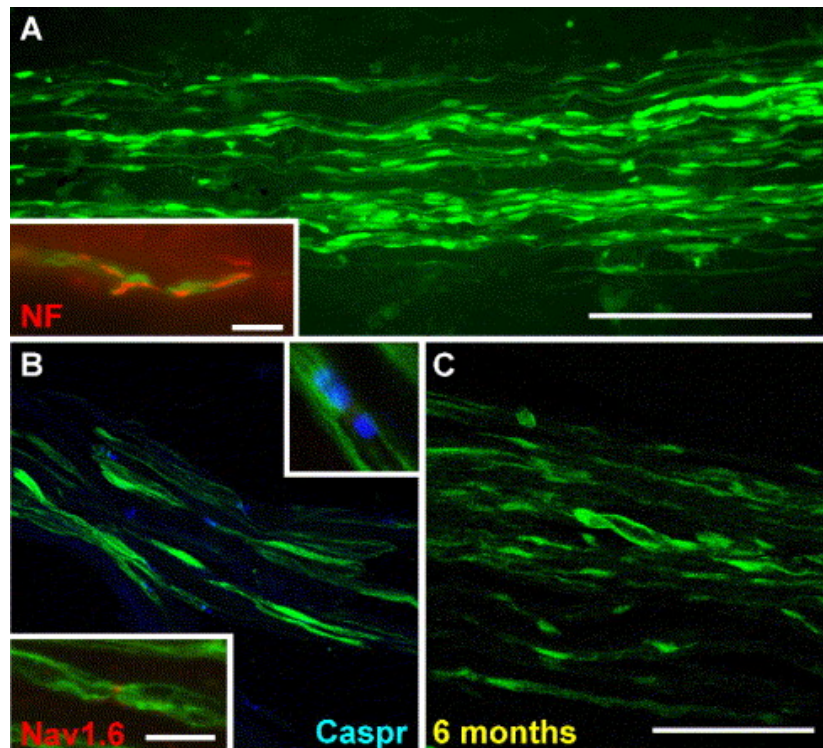
Olfactory ensheathing cell (OEC) transplantation into crushed sciatic nerve

Fluorescent images of living sciatic nerve whole mount taken immediately upon extraction and desheathment (A), and fixed nerve at 5 weeks post transplantation (B). Green fluorescent protein (GFP)-OECs are distributed longitudinally several millimeters from the injection site. Areas of increased fluorescence indicate the nuclei and dense cytoplasmic regions of the OECs. Higher magnification image of teased, fixed nerve taken immediately upon removal at 5 weeks post transplantation (C1). GFP-OECs associate with individual axons within the lesion site. An individual living axon with putative nodes of Ranvier (boxed) and areas of concentrated fluorescence, which indicate OEC nuclei and cytoplasm (C2).

Scale bar: A=500 μ m, B = 25 μ m, C1= 11 μ m, C2=3.5 μ m

Figure 3

Image A taken by Mary Dombrowski. Image B and C taken by Dr. Masanori Sasaki.

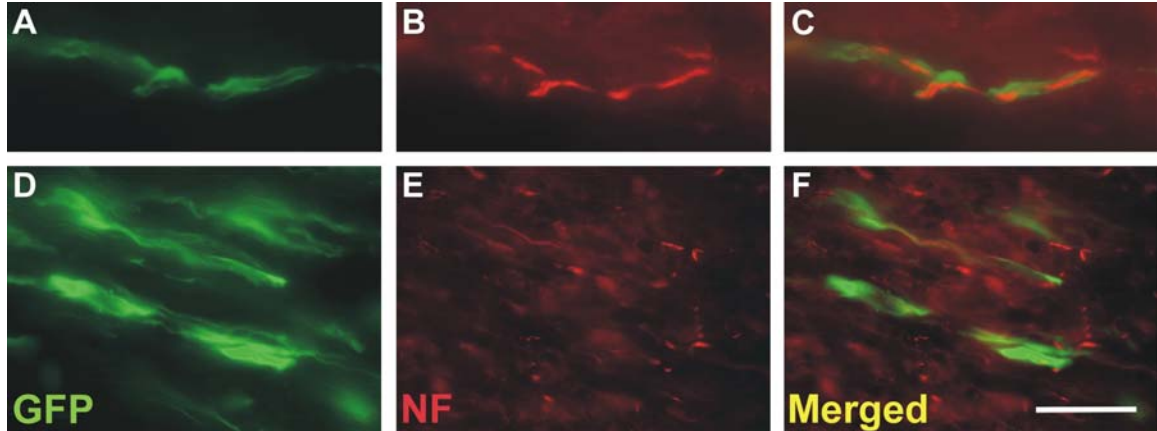


**Confocal images at 5 weeks and 6 months post OEC transplantation
of the crushed sciatic nerve.**

This image demonstrates an abundance of GFP-OECs distributed longitudinally in the injured nerve (A). Association of GFP fluorescence with axonal neurofilament staining (Inset A). Caspr immunostaining demonstrates GFP-OECs in the transplanted region and the inset in the upper right is a higher magnification of individual nodal regions (B). Immunostaining for sodium channel $Na_v1.6$ reveals nodal regions within the crush lesion site (B). Six months post crush injury and transplantation the GFP-OECs do survive (C).

Scale bar: A = 200 μ m, B = 40 μ m, C = 80 μ m, D= 13 μ m, A inset=13 μ m

Figure 4



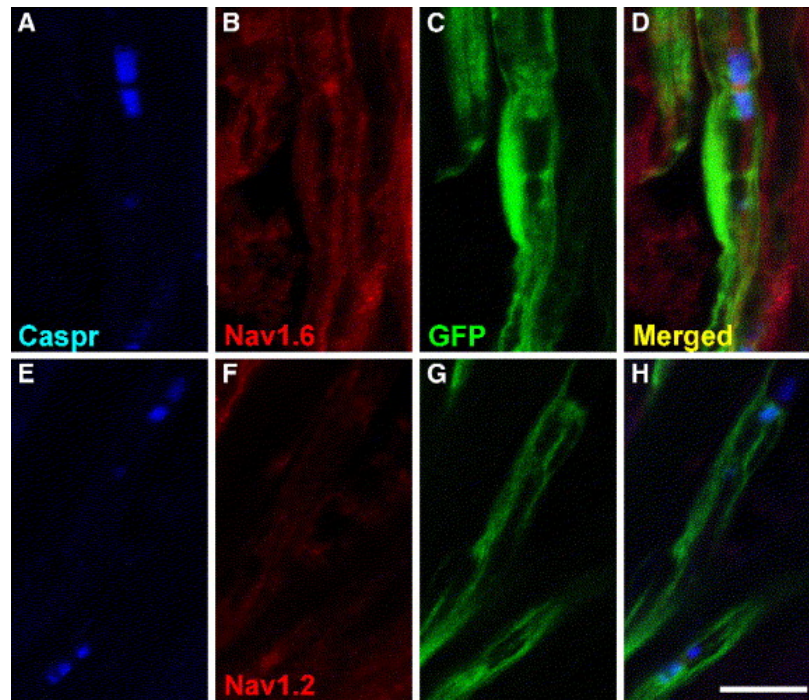
Neurofilament staining four weeks post GFP-OEC transplantation

These images demonstrate GFP immunofluorescence four weeks post GFP-OEC transplantation into the sciatic nerve crush injury (A, D). Immunostaining for neurofilament (NF) of the same field (B, E). Merged images show GFP fluorescence surrounding the NF stained axon (C,F).

Scale bar A-C = 20 μm , D-F = 40 μm

Figure 5

Images taken by Dr. Masanori Sasaki.



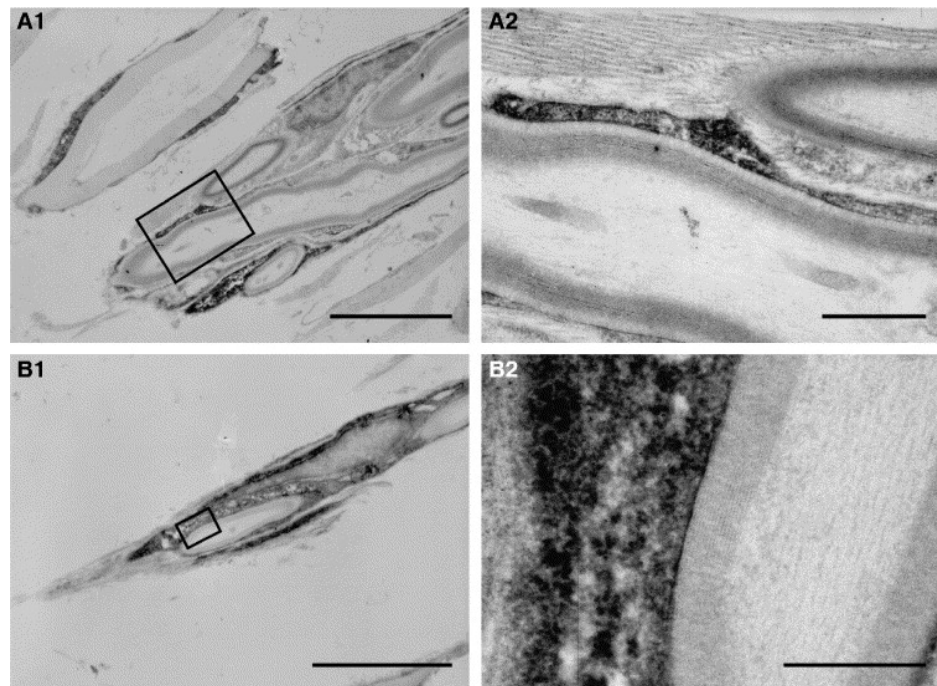
Na_v1.2 and Na_v1.6 at GFP-OEC nodes in crushed sciatic nerve

At 5 weeks after transplantation, Caspr immunostaining flanks the node of Ranvier (A) formed by GFP-OECs (C). Na_v1.6 clustering is displayed at most Caspr-delimited nodes (B). In contrast, Na_v1.2 is not seen, indicating node maturity (F). GFP-OECs at 5 weeks post transplant (G) and corresponding Caspr immunostaining (E). Merged images of (A–C) and (E–G) are shown in (D) and (H), respectively.

Scale bars A-H = 10 μm

Figure 6

Images taken by Dr. Masanori Sasaki.



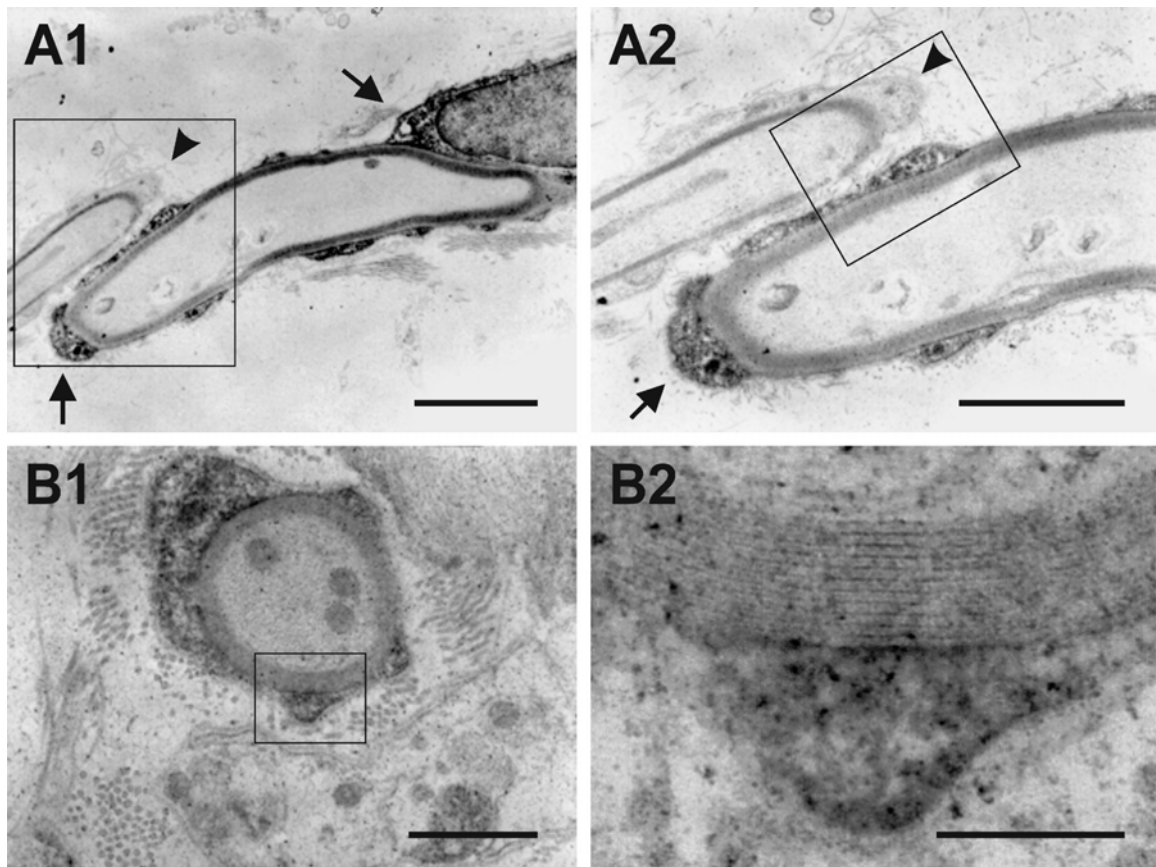
Electron micrographs of anti-GFP immunoperoxidase staining

At 7 weeks post transplantation reaction product can be seen in the cytoplasm of the GFP-OECs, which have formed myelin around an axon (A1). Higher magnification of boxed area in A1 (A2). Another myelinated axon showing intense reaction product in the myelin forming cell (B1). Higher magnification of boxed area in B1 (B2).

Scale bar A1=5 μ m, A2=1 μ m, B1=1 μ m, B2=0.5 μ m

Figure 7

Images taken and modified by Dr. Masanori Sasaki (5).



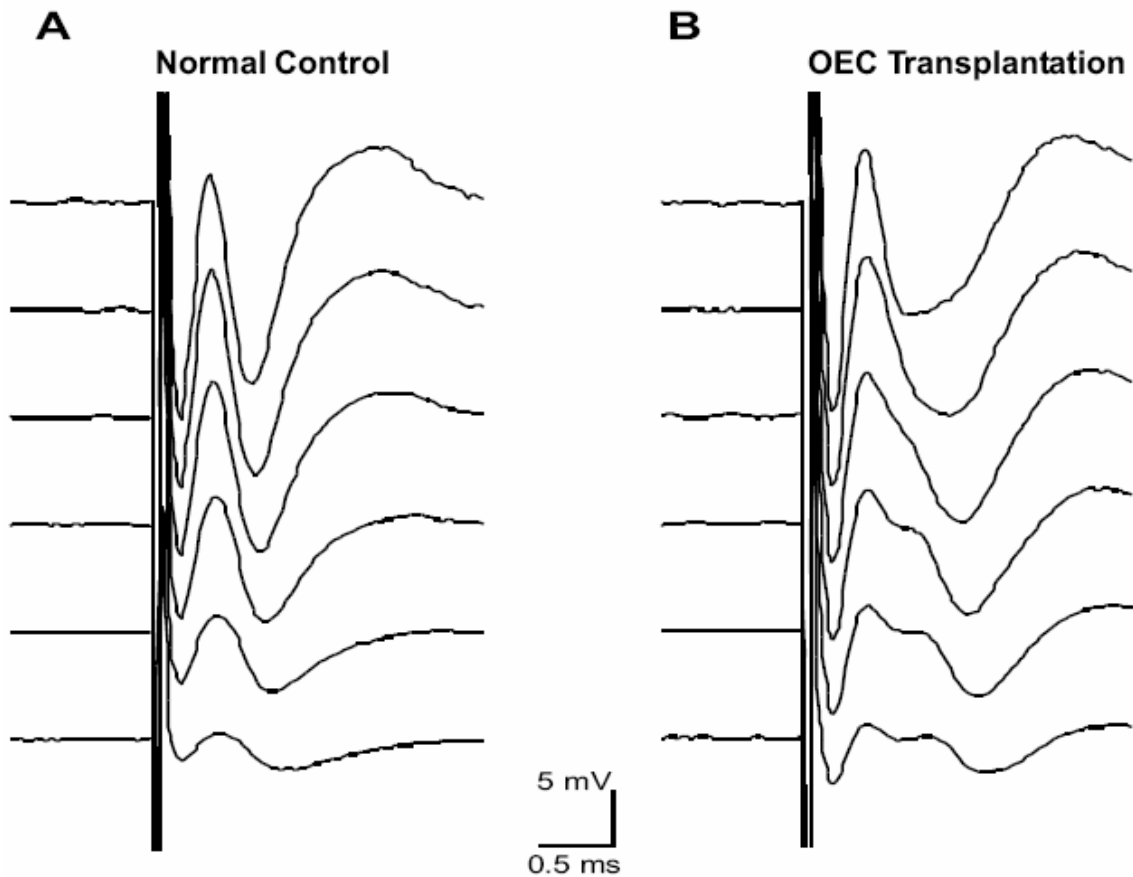
**Electron micrograph images of transplanted sciatic nerve with
anti-GFP immunoperoxidase**

Reaction product for anti-GFP is seen in the cytoplasm of myelin forming cells, but not directly in the myelin (A1). In this same figure a myelinated axon not containing any reaction product is also seen. The boxed area of A1 (A2). An axonal cross section demonstrates cytoplasmic reaction product (B1). The boxed area of B1 (B2).

Scale bars A1 = 5 μm , A2 = 2 μm , B1 = 1 μm , B2 = 0.25 μm .

Figure 8

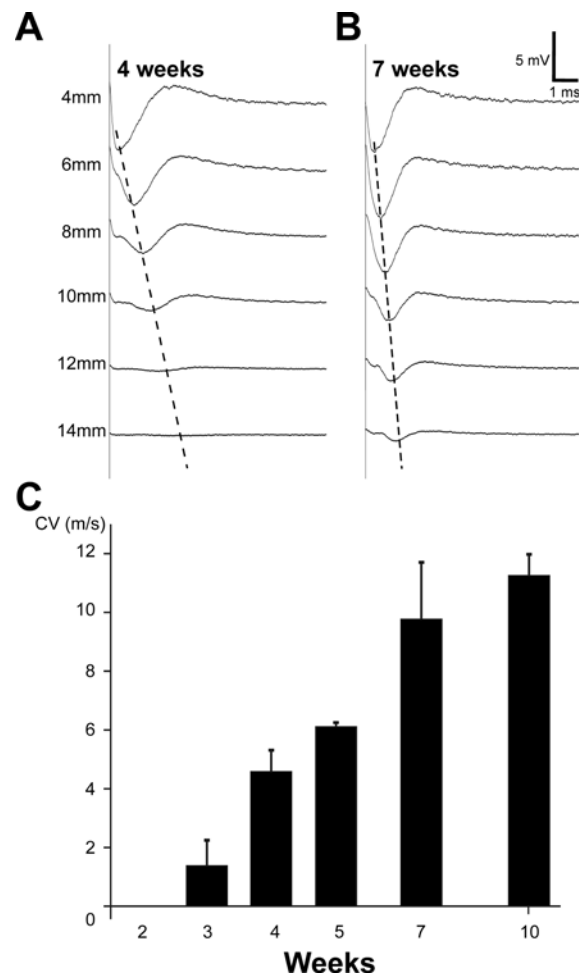
Images produced by Dr. Hajime Tokuno.



**Electrophysiology studies of sciatic nerve segment 8 weeks post
GFP-OEC transplant**

The figure on the left represents the compound action potentials (CAP) of the sciatic nerve of normal control animals. The figure on the right represents the CAPs of transplanted hosts eight weeks following OEC transplantation. The conduction velocity of the normal, control nerve was 44.8 m/s, while the conduction velocity of the crushed nerve was 25.6 m/s.

Figure 9



Electrophysiology studies of GFP-OEC transplanted sciatic nerve at varying time intervals

Compound action potential (CAP) at a specific distance away from the stimulus electrode (A, B). Dashed lines indicate peak negativity of CAP. It can be determined that at four weeks post sciatic nerve injury and OEC transplantation, the CAP signal returns. At seven weeks post sciatic nerve injury and OEC transplantation, there is shortening of the peak latencies and improvement in the CAP amplitude over a greater distance (B). Over 10 weeks increasing conduction velocity was observed (n=4 per week) (C).

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