Regrowth of rabbit optic nerve fibers after transection and peripheral nerve graft : an electron microscopic study

by Marie Elise Legare Myron

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences
Montana State University

Abstract:
Nerve fibers in the central nervous system (CNS) have been generally reported not to regenerate after injury whereas those in the peripheral nervous system (PNS) do. It is therefore of interest to study the effects of implanting peripheral nerve tissue into damaged CNS to see if such grafts might have a positive effect on CNS regrowth. A four to five mm section of autologous sciatic nerve (PNS) was grafted between the transected ends of rabbit optic nerve (CNS). Tissue was then examined at 1,2,3,4,6,7, and 10 weeks post-operatively for evidence of nerve fiber regrowth, using light and electron microscopy. Results were compared with non-grafted transected optic nerve. In the non-grafted tissues, the optic nerve fibers underwent an abortive attempt at regeneration and did not appear to cross the lesion, leaving a "nerve" primarily composed of glial scar. In the grafted tissues, the PNS elements grew into the proximal and distal ends of the transected area and elongating nerve fibers were seen on both sides of the lesion. Subsequently, these fibers increased in size and were surrounded by normal PNS-type myelin sheathing. Thus, the use of a peripheral nerve graft appears to promote transected CNS nerve fiber growth, maturation, and myelination.

REGROWTH OF RABBIT OPTIC NERVE FIBERS AFTER TRANSECTION AND PERIPHERAL NERVE GRAFT: AN ELECTRON MICROSCOPIC STUDY

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ABSTRACT

Nerve fibers in the central nervous system (CNS) have been generally reported not to regenerate after injury whereas those in the peripheral nervous system (PNS) do. It is therefore of interest to study the effects of implanting peripheral nerve tissue into damaged CNS to see if such grafts might have a positive effect on CNS regrowth. A four to five mm section of autologous sciatic nerve (PNS) was grafted between the transected ends of rabbit optic nerve (CNS). Tissue was then examined at 1,2,3,4,6,7, and 10 weeks post-operatively for evidence of nerve fiber regrowth, using light and electron microscopy. Results were compared with non-grafted transected optic nerve. In the non-grafted tissues, the optic nerve fibers underwent an abortive attempt at regeneration and did not appear to cross the lesion, leaving a "nerve" primarily composed of glial scar. In the grafted tissues, the PNS elements grew into the proximal and distal ends of the transected area and elongating nerve fibers were seen on both sides of the lesion. Subsequently, these fibers increased in size and were surrounded by normal PNS-type myelin sheathing. Thus, the use of a peripheral nerve graft appears to promote transected CNS nerve fiber growth, maturation, and myelination.

INTRODUCTION

Most animals have the capacity for regeneration of damaged nerve cell processes in their embryonic form (Piah,'55; Stephens,'59). Lower vertebrates, such as fish and amphibians, retain this capacity for functional axonal regrowth throughout adulthood (Kerr,'75; Singer et al.,'79; Turner and Singer,'74). However, the ability to achieve functional restitution of damaged regenerating fibers appears to be limited to only specific nerve fibers under certain conditions in mature reptiles and mammals (Flint and Berry,'73; Hess,'56; Hooker and Nicholas,'30; McMasters,'62).

In the early 1920's, Ramon y Cajal described the process of axonal regeneration in both the peripheral and central nervous systems of mammals (Cajal, ref. in David and Aguayo,'81). Cajal maintained that in the central nervous system (CNS) fibers underwent an abortive attempt at regeneration, with neither complete anatomical nor functional restitution occurring. He attributed this failure to the lack of appropriate "pathways" and neurotrophic substances necessary for the growing axons (Cajal, ref. in Sugar and Gerard,'40). This concept was considered dogma for many decades with the negative results from later experiments (Hess,'56; Hooker and Nicholas,'30; Osterberg and Wallenberg,'62),

adding credence to the hopelessness of CNS regeneration in mammals. On the other hand, it has long been known that mammalian peripheral nerves are capable of reinnervating an injured area, given that the local tissue environment is conducive to growth (Sanders,'42; Young,'42).

Studies done in the 1920's and '30's maintained that Schwann cells were not essential for fiber regeneration in peripheral nerve tissues (Harrison,'24; Spiedel,'32; Weiss,'34), since axons grown in culture were observed to elongate in the absence of connective tissue cells. More recently, the regenerative capabilities of mammalian peripheral fibers have been, for the most part, attributed to factors associated with the supporting cells of Schwann (Bernstein and Bernstein, '71; Marx,'80; Richardson et al.,'80; Varon,'77). Within hours after injury to a peripheral nerve fiber, Schwann cells elongate and migrate to position themselves end to end (Holmes and Young,'40; Allt,'76). These cellular tubes bridge the forming connective tissue scar and effectively establish a route for sprouting nerve fibers to follow from the proximal to distal stumps of the injured nerve (Richardson et al.,'80). The growing axons enter the Schwann cell tubes in the degenerating distal nerve stump and gener-

ally regrow to an appropriate terminal location (Allt,'76). The Schwann cells begin to form new myelin for the axon within three weeks after the injury (Allt,'76). Varon ('77) and Osterberg ('62) have suggested that perhaps there are unknown intrinsic factors associated with the surrounding Schwann cells or connective tissue, possibly of a chemical nature, that could play important roles in establishing the proper environment for regeneration. Similarly, it has been demonstrated that the presence of both degenerating neural tissue and proliferating glial cells plays an important role in the successful sprouting of fibers in the central nervous system (Campbell and Windle,'60; Raymond and Levine,'81).

Transected axons from mammalian spinal cord begin to elongate (Richardson et al.,'80; Richardson et al.,'82), just as do peripheral axons, but after a short period of time they cease to grow (Bernstein and Bernstein,'71). A scar composed of a tangled mass of neuroglia (Marx,'80), degenerating axons, connective tissue elements, and cavitations (Kao et al.,'77a) apparently blocks their growth (Clemente and Windle,'54; Windle and Chambers,'50). Cavitations arise from a process of tissue autolysis occuring from the release of lysosomal hydrolases by the severed axons (Kao and

Chang,'77). This tissue-fluid interface is an area that the growing axons must traverse without the guidance of cellular elements. Successful regrowth has been only rarely reported in experimental situations (Sugar and Gerard,'40), and many of these experiments have been questioned after unsuccessful attempts at repetition (Barnard nd Carpenter,'49). There is some question as to whether an inhospitable environmental response is the only factor causing unsuccessful CNS axonal regneration or whether perhaps CNS neurons are intrinsically less capable of regeneration than PNS neurons (Richardson et al.,'82).

Windle (Guth and Windle,'73) demonstrated that when scar tissue formation was inhibited with the use of pharmacological agents, neural sprouts continued to grow for extended periods of time. However, these elongating fibers still did not establish the appropriate synaptic contacts. If this ability for neuronal regrowth appears to be an intrinsic (inherent) property of all damaged neurons, then the surrounding environment must determine whether or not regeneration will be abortive or successful.

It was found during the late 1970's that damaged peripheral axons with a known capacity to regenerate failed to elongate for more than a few millimeters when

placed in a milieu of central glial cells (Aguayo et al.,'79; Weinberg and Spencer,'79). On the other hand, it has been shown that CNS axons will grow into peripheral nerve segments transplanted into spinal cords (Blakemore,'77; Richardson et al.,'80; Weinberg and Spencer,'79). Theoretically, this reconstruction gave the CNS fibers contact with the elements of the peripheral environment which seem to be so conducive to successful regenerative growth.

Kao ('70, '74) and Kao et al. ('77), grafted pieces of autologous sciatic nerve into the injury site of transected mam malian spinal cord and demonstrated by both light and electron microscopy that both the initial tissue-fluid interface and the formation of a connective tissue scar were minimized. Thus, gross structural continuity of the graft with the spinal cord stumps was acheived. Subsequent electron micrographic studies showed axons passing between the two spinal cord stumps via the sciatic nerve grafts (Kao,'74; Kao et al.,'77). These and other experiments (Benfey and Aguayo,'82; David and Aguayo,'81; Richardson et al.,'80; Richardson et al.,'82b) are challenging the long-held concepts regarding the inability of CNS fibers to regenerate.

Unfortunately, although spinal cord regeneration has many practical implications, the spinal cord may not

be the most useful model for studying CNS regeneration. The spinal cord is an extremely complex tissue with numerous groups of axons, both afferent and efferent, traveling to many different central and peripheral terminations. The spinal cord is extremely sensitive to trauma and is susceptible to postraumatic ischemia and necrosis (Griffiths,'76; Rivlin and Tator,'78). During surgery, it is often difficult to accurately verify whether or not the cord has been totally transected (Puchala and Windle,'77). In experiments utilizing local crush as the source of trauma, there is no way of being certain that all of the fibers have been affected (Nathanial and Pease,'63). Accurate determination of the origins of fibers seen after a period of growth is difficult. They may be regenerated axons or perhaps collateral sprouts from neighboring axons that were never damaged in the intital injury (Bernstein and Bernstein,'71; Goldberger and Murray,'74; Liu and Chambers,'58; Murray and Goldberger,'74).

The experiments reported here were designed to morphologically examine the possibility of regeneration after grafting peripheral nerve into a simpler model of damaged mammalian CNS. Theoretically, the graft should minimize glial scarring and also provide a more favorable physical and chemical environment for the growing

fibers. To minimize some of the problems incurred when working with spinal cord transections, the optic nerve was chosen for study. The optic nerve is surgically accessible extracranially, clearly defined in the rabbit, and is thought to be mainly composed of retinal ganglion cell axons (Vaney and Hughes,'76). A strong dural sheath of heavy connective tissue permits attachment of a graft to transected stumps with the use of sutures. Accurate determination of the morphological processes occuring after transection and grafting should therefore be possible, and be much simpler to interpret than are similar experiments done in spinal cord.

MATERIALS AND METHODS

Experimental Animals

Male, New Zealand white rabbits, 1.7 Kg to 4.8 Kg in weight were used for the experiments. They ranged in age from 5 to 10 weeks.

Anesthesia

A solution of sodium pentobarbitol was used as anesthetic for both surgery and for sacrificing the animal at the end of the experiment. It was injected into the marginal ear vein at an initial dose of 30 mg/Kg body weight. Successive doses were given in 10 mg increments until the pinch of the Achilles tendon produced no reflex withdrawal of the limb. During the surgeries, the tendon reflex was often retested and additional anesthetic was administered as needed.

Surgical Procedures

The left orbital roof was exposed by making an 8 cm midline incision and by reflecting the subcutaneous and muscle tissues. The bony orbital roof was then removed with rongeurs, with care being taken not to leave rough edges which could injure soft tissues. Scissors were used to separate and cut away the individual connective tissue and muscular layers superior and posterior to the eye itself. The attachment of the superior rectus tendon to the eye was kept intact and grasped with a

hemostat. This allowed maintenance of a steady forward and downward traction on the eye. Bleeding from the scleral vessels was controlled by cautery. Isotonic saline wash was utilized to prevent drying of the sclera and associated tissues.

Approximately 6 mm of the optic nerve was exposed and the adjacent fascia and clotted blood were removed. Care was taken to minimize any disturbance of the nerve's blood supply or the venous sinuses in the orbit (McConnell,'64). An anchoring suture was passed through the portion of the exposed nerve closest to the brain using 8 0 silk with a GS 10 needle. Another suture was passed beneath this portion of the nerve and tied to adjacent tissues to minimize retraction of the nerve after transection. The optic nerve was then cut distal to the anchoring sutures and 1.5 to 2.0 mm from the back of the eye with sharp iris scissors. In those animals used as controls (transected but not grafted), the two optic nerve stumps were reopposed with two to four separate sutures. Care was taken to minimize folding and gathering of the abutted nerves while still achieving as complete an apposition as possible. In the grafted animals, the sciatic nerve was exposed high in the right popliteal fossa and a 5mm section of nerve was removed with scissors. The nerve section was

covered with isotonic saline in a petri dish and excessive connective tissue was trimmed away. The sciatic nerve section was then aligned end to end between the two stumps of the severed optic nerve and sutured in place using the same techniques as in the non-grafted animals.

After suturing, muscle and connective tissue planes were returned over the nerve and the eye. Subcutaneous tissues and the skin incision were then closed with sutures. A 0.25 ml injection of a combination antibiotic (1.0 ml = 200,000 units procaine, penicillin G, and dihydrostreptomycin sulfate) was given intramuscularly and injections were continued at 0.1 ml per day for seven to ten days postoperatively.

Sacrifice and Tissue Preparation

Experimental animals were sacrificed at 1, 2, 3, 4, 6, 7, and 10 weeks post-operatively while control animals were sacrificed at 2 and 8 weeks. Anesthetized animals were perfused with a solution of 3% glutaraldehyde in a 0.1M phosphate buffer (pH 7.3) using the following procedures.

A subcostal incision was made and the xiphoid process of the sternum was held with forceps allowing manipulation of the anterior chest wall. A midline incision through the abdominal wall was made to provide

a release for intraabdominal pressure caused by muscular contraction during perfusion. Incisions were made across the diaphragm and along the midlateral extent of the rib cage. The anterior rib cage was then lifted superiorly with a hemostat which simultaneously clamped the internal thoracic arteries. The pericardial sac was immediately opened and a 13-gauge needle was inserted into the left ventricle and upward into the aorta. The right atrium was immediately cut and the fixative allowed to flow under 100-120 mm Hg pressure for 10-15 minutes.

Following perfusion, the eye and approximately 10 mm of optic nerve, which included the graft in experimental animals, were exposed and removed. Scar tissue surrounding the nerve was dissected away. Normal undisturbed optic nerve tissue from the opposite side was also removed in some animals for comparison with the experimental tissues. The nerve was then sectioned into 2 to 2.5 mm segments. The segments were labelled according to their relative positions, from the most distal (closest to the brain) to the most proximal (closest to the eye). The tissues were further diced into 1 mm^3 pieces and placed in fixative for 2-3 hours. After 4 to 5 rinses in buffer wash (10-15 minutes per rinse), tissues were left overnight in buffer. A sol-

ution of 1% OsO_4 in 0.1M phosphate buffer was used to postfix the tissues for 2 to 2.5 hours. They were then dehydrated through a series of increasingly concentrated ethanol solutions (% to %), followed by propylene oxide. Tissues were infiltrated, with rotation, for 1 to 1.5 hours in a 1:1 mixture of propylene oxide and Maraglas (Freeman and Spurlock,'62), followed by a 1:4 mixture for 30 minutes. Tissues were then infiltrated with 100% Maraglas for two one-hour periods. The infiltrated tissues were embedded in #00 plastic capsules filled with 100% Maraglas then polymerized in a 60°C vacuum oven for 72-96 hours.

The dehydration steps for two of the experiments were altered in the following manner. The ethanol and propylene oxide steps were replaced by immersion of fixed tissues into activated 2,2-dimethoxypropane for 30 minutes (Thorpe and Harvey,'79). No differences were found in the tissues utilizing this method.

For light microscopy, two micron sections were cut on glass knives using an LKB 8800 Ultratome III, then stained with toluidine blue in borax (Meek,'76). These sections were studied for tissue analysis as well as for orientation for thin sectioning. Thin sections suitable for electron microscopy were cut on the LKB 8800 Ultratome III using glass knives, and then mounted on

uncoated copper grids. The sections were post-stained in lead citrate (Venable and Coggeshall,'65) and saturated uranyl acetate (Watson,'58). Thin sections were studied and photographed in a Zeiss EM9S-2 electron microscope.

Criterion used for distinguishing the following cells were similar to those described in the literature: Growth cones (Bunge,'73; Kawana,'71; Yamada,'71), glial cells (Bignami and Ralston,'69; Phillips,'73; Vaughn and Pease,'70; Wuerker,'70), and axons (Peters et al.,'76; Wuerker,'70).

RESULTS

Unoperated Optic Nerve

The optic nerve in unoperated control tissue was composed of myelinated axons with diameters ranging from 0.8 to 2.0 um (um = micrometers), with most of the axons in the range of 1.2 to 1.5 um (see fig. 1, all figures are located in the appendix). The myelin sheaths generally contained 8 to 10 dense period lines with a periodicity of 150 A. Axoplasmic constuitents included neurofilaments (100 A diameter), microtubules (250 A diameter), mitochondria, and dilated, membrane bounded vesicles (750 to 2300 A in diameter) which were characteristically rounded or oval. Unmyelinated fibers were only rarely found within the sections examined, and were most likely examples of nodal areas. Other studies (Vaney,'76) have reported the rabbit optic nerve to be composed of at least 98% myelinated, small diametered axons.

The glial environment was composed predominantly of astrocytic processes which were interwoven with neuronal elements and formed the limiting membranes (fig. 3 and 4). Astrocytes were identified by numerous filaments (80 to 90 A in diameter) running parallel to the long axis of the processes and by their scattered glycogen granules (350 A in diameter). Scattered elongated

mitochondria were found within the processes. Microtubules were rarely seen. The cytoplasm was generally electron lucid relative to other glial cells. Astrocytic nuclei (fig. 2) were irregular or bean shaped and the nuclear membrane was often thrown into folds. Nuclear chromatin generally was uniformly distributed except for slight condensations beneath the nuclear membrane.

Although not as predominant as astrocytes, oligodendrocytes were found scattered amid the groups of myelinated fibers. Oligodendrocyte nuclei were more round or oval than those of astrocytes and more frequently contained clumped chromatin. The cytoplasm was fairly dense and contained a large number of ribosomes, well developed rough endoplasmic reticulum (RER), and a prominant Golgi apparatus (fig. 3). Abundant mitochondria were also present but tended to be inconspicuous due to the density of the cytoplasm. Oligodendrocytes lacked the filaments and glycogen found in astrocytes but microtubules could sometimes be found in their cytoplasm.

Glial limiting membranes (fig. 4) were composed of tightly packed astrocytic processes separated from collagen and other connective tissue elements by a heavy basal lamina 500 to 600 A thick. A cytoplasmic conden-

sation, 400 A thick was found immediately beneath the plasma membrane deep to the basal lamina. It was similar to that seen at cell to cell junctions but was not associated with other areas of astrocytic cell membrane.

Grafted Experimental Nerve

In the grafted tissue, sutures were identified by light microscopy and used as reference points to localize the original graft-optic nerve interfaces. Segments were termed either proximal (closer to the ganglion cell bodies), graft (within the boundaries of the two sutured areas), or distal (closer to the brain). These designations are used throughout the following descriptions in reference to the position of the sections examined.

One Week

LIGHT MICROSCOPY - Proximal to the original graft-optic nerve interface, most of the optic nerve appeared normal (fig. 5). Close to the interface, the tissue had a "foamy" appearance due to extreme vacuolization.

In the grafted peripheral nerve segments (figs. 6 and 7), myelin degeneration was very evident. Because of the wide range of fiber diameters, the thickened myelin surrounding these fibers, and the abundant con-

nective tissue, it was not difficult to distinguish this peripheral nerve tissue from the original optic nerve.

Distal to the graft, the optic nerve generally appeared normal except for scattered instances of swollen myelin. Connective tissue elements, as in controls, were not seen except in association with external and vascular limiting membranes.

ELECTRON MICROSCOPY - In tissue segments proximal to the graft there were many examples of degenerating CNS myelin which had either a loosely wrapped or a homogenous dense appearance with no observable fine structure (fig. 12). Degenerating axons were also evident in which the axoplasm was obviously vesiculated or in other instances contained a homogenous granular material (fig. 12).

Astrocytic cell bodies and processes were similar to controls except for increased amounts of glycogen.

Many of the myelinated fibers found in central nervous tissue proximal to the graft appeared typical in structure (figs. 9 and 10). These fibers had diameters ranging from 0.25 to 1.2 um and their myelin structure and thickness were similar to those of controls. Characteristic microtubules, neurofilaments, occasional mitochondria and dilated vesicles were in the axoplasm.

Growing nerve fibers apparently involved in the complete series of changes related to myelination were found (figs. 9, 10, and 11). Unlike unoperated tissues, there were unmyelinated fibers, 0.25 to 0.35 um in diameter, that lacked individual sheaths. Some axons were partially to totally encircled by thin cytoplasmic processes while others had loosely arranged multiple wrappings of these cytoplasmic processes. In some fibers, the inner adaxonal wrap was composed of a thickened cytoplasmic process but the outer wraps were of compacted myelin. These cytoplasmic processes had an electron density comparable to those of astrocytes and they contained a paucity of organelles. It was difficult to trace the processes to continuity with cell bodies but they were similar to active oligodendrocyte processes described in developing CNS tissues (Phillips,'73).

Close to the original graft-optic nerve interface, CNS myelinated fibers were found within an altered glial environment. These fibers were generally within the smaller range of fibers in control tissue and their myelin structure and thickness were similar to that of controls. There appeared to be more astrocytic processes per area than in controls. Astrocytic and oligodendroglial cell bodies were similar in structure to

those previously described but were more abundant. Microglial-like cells (fig. 13) contained obvious lipid inclusions and degenerating myelin figures, as well as typical ribosomes, mitochondria, and RER. The general nuclear and cytoplasmic morphology of these cells was similar to oligodendrocytes but the presence of degenerating myelin and abundant lipid in the cytoplasm indicated a phagocytic role. Some areas showed indications of total degeneration of neuronal elements with scattered profiles of glial elements present (fig. 17).

Glial limiting membranes (fig. 37) were seen at optic nerve-connective tissue interfaces, similar to controls but having a more convoluted border composed of more glial processes than normal.

Examples of peripheral nerve tissue similar to tissue in the graft, were found in proximal segments. Such tissues were easily characterized by the abundant collagen present in the extracellular spaces and the basal lamina around individual cells. The associated Schwann cells (fig. 14) had a dense cytoplasm which contained ribosomes, RER, mitochondria, Golgi apparatus, pinocytotic vesicles, lipid inclusions, and myelin debris. The Schwann cells sometimes had thin, highly folded cytoplasmic processes which interdigitated with each other. Their nuclei contained compacted

chromatin and nucleoli were often apparent.

Degenerating PNS myelin was characteristically found as large diameter (1.5 to 4.5 um) globules (fig. 15) contained within Schwann cell cytoplasm. Myelin within the globules was extremely fragmented, with some areas retaining evidence of normal periodicity while other areas had a uniform lipid-like appearance. Only rarely were axons seen within the degenerating myelin, but when present they were swollen and extremely vesiculated.

A few small axons (fig. 16) were present in the connective tissue space between the large phagocytic Schwann cells. They were devoid of a Schwann cell sheath but were surrounded by a basal lamina. These axons were approximately 0.5 um in diameter and contained normal axoplasmic constituents.

The grafted segments were composed entirely of PNS degenerating tissue similar to that just described as being present in proximal segments.

Distal to the graft, the nerve was composed entirely of CNS tissue with apparent myelin degeneration similar to that described in proximal sections (figs. 12 and 13). Occasionally, myelinated fibers that were smaller (0.7 to 1.1 um in diameter) than those normally present in unoperated nerves were observed. Their

associated myelin structure and axoplasmic constituents were normal.

Two Weeks

LIGHT MICROSCOPY - As at one week, the central nervous tissue proximal to the original graft-optic nerve interface was consistently vacuolated (fig. 18) and contained relatively more glial cell nuclei than was seen in controls (fig. 19). Peripheral nerve type tissues were more evident than in the samples taken at one week. Such invading peripheral nerve tissue was characterized by large myelin figures and connective tissue elements which separated groups of fibers into discreet bundles (fig. 20).

Tissue close to the graft-optic nerve interface was extremely vacuolated and phagocytic cell nuclei were apparent. Due to the extreme degeneration, it was difficult to differentiate optic nerve from peripheral graft. Degenerated tissue was "walled off" into discreet compartments by surrounding connective tissue elements. Blood vessels appeared to have an unusual amount of connective tissue surrounding them.

Distal to the graft, optic nerve fibers generally appeared somewhat fragmented (fig. 21) with scattered instances of myelin degeneration and a few smaller than normal myelinated fibers present.

ELECTRON MICROSCOPY - Proximal segments that were primarily glial "scar" were composed predominantly of astrocytic processes and abundant degenerating myelin (fig. 22). Healthy appearing axons were generally not found in such tissue.

The majority of the tissues in the proximal segments was of peripheral origin and was characterized by abundant collagen, Schwann cells, and basal laminae surrounding the cellular elements. Phagocytic activity of Schwann cells similar to that described in animals sacrificed at one week was evident.

This peripheral nerve environment contained many nerve fibers which had the characteristics usually associated with growing axons and which were not associated with an ensheathing cell (figs. 23 and 24). They had normal axoplasmic constituents and were often surrounded by a redundantly folded basal lamina (figs. 24 and 33). The diameters of these unsheathed fibers ranged from 0.4 to 1.6 um. Occasionally, these fibers were found in close proximity to cytoplasmic extensions of Schwann cells. The cytoplasm of these Schwann cell extensions was often packed with numerous ribosomal rosettes. Occasional Growth cones (fig. 25) were found which contained an abundance of organelles including ribosomes, smooth endoplasmic reticulum (SER),

mitochondria, microtubules, and filaments.

Nerve fibers or growth cones were never found in the peripheral tissue within the graft at two weeks. Such tissues contained many Schwann cells (fig. 26) that appeared to be actively involved in phagocytic activity and frequently contained small packets of debris just deep to the membrane.

The few areas with CNS-type tissues located close to the graft-optic nerve interface were generally composed of extremely deteriorated elements identical to degenerating optic nerve described in the one week animal. Occasionaly, myelin with normal periodicity was found in association with either an empty vesiculated axon or a shrunken axon with granulated cytoplasm. Myelinated axons (approximately 0.7 um in diameter) were rarely found. Increased amounts of glycogen were found in astrocytic processes and glial cell nuclei seemed to be more apparent than in control tissues. The glial limiting membranes observed at connective tissue-glial interfaces were similar to those described previously.

Degenerating optic nerve in distal segments, was similar to that described previously except that the majority of fibers contained a dense granulated cytoplasm. Degenerating tissue was sometimes found extracellularly, in such cases there was minimal phagocytic

activity. Again, normal appearing myelin was often observed surrounding an axon that was obviously degenerating. A few small myelinated fibers, with diameters of 0.6 to 0.9 um (smaller than the average in controls) were present. Segments of the axons appeared normal and contained characteristic cytoplasmic organelles, while other segments within the same axon were undergoing vesiculation and degeneration.

Rarely, connective tissues were found apposed to a glial limiting membrane in the degenerating optic nerve distal to the lesion area. Nerve fibers or degenerating peripheral nerve were never found within these connective tissues.

Three Weeks

LIGHT MICROSCOPY - In proximal nerve segments the tissue was vacuolated and undergoing degeneration. Peripheral nerve tissue was common and identified by large bundles of degenerating myelin with a wide range of diameters and abundant associated connective tissue (figs. 28 and 29). Scarred optic nerve areas were characterized by containing more glial processes than did nerve fibers.

Close to, and within the graft, there were areas of extreme vacuolization and with such abundant myelin debris that the tissue was difficult to identify either as original optic nerve or as peripheral graft (fig.

27). Phagocytic cells were apparent throughout the sections examined.

Distal to the graft, degenerating optic nerve tissues (fig. 30) were characterized by a general "swelling" of the myelin, fragmented fibers, and more glial nuclei than usual. A thickened connective tissue layer was found encircling vascular elements.

ELECTRON MICROSCOPY - In proximal segments the nerve was composed almost entirely of tissues derived from peripheral nerve. Many small unsheathed axons (figs. 31, 32, and 33) were found (0.3 to 1.5 um in diameter) amid collagen fibers. Such axons were surrounded by a highly folded basal lamina (fig. 33), and were sometimes associated with Schwann cell extensions (figs. 32 and 34). A few fibers were found apposed to other unsheathed axons with no significant basal lamina between them (fig. 36). Characteristic organelles were found within the axoplasm. Schwann cell extensions were identified by the abundant ribosomal rosettes within the cytoplasm.

Growth cones (fig. 35), 2.9 to 3.4 um in diameter, were occasionally found. Cytoplasmic constituents were similar to that described at two weeks and in addition, occasionally included a membrane-bounded C-shaped structure (fig. 35-inset) with finely granular content,

reported to be characteristic of axonal growth cones (Bunge,'73).

In the scattered areas of proximal segments composed of CNS derived tissues, astrocytic processes were found to have a greater amount of glycogen than normal. Phagocytic activity appeared to be minimal although an abundance of degenerating myelin was located in the extracellular spaces of the tissue. Rarely, small myelinated fibers were observed in this degenerated tissue.

Glial limiting membranes (fig. 37) were similar to those described previously in grafted experimental tissues.

Close to and within the graft area, many unsheathed fibers were observed in a peripheral environment that was filled with phagocytic cells containing degenerating peripheral myelin. Highly folded basal laminae surrounded the unsheathed fibers and a few fibers were closely apposed to Schwann cell cytoplasmic extensions.

Astrocytic processes, containing more glycogen than normal, composed most of the tissue distal to the graft. Examples of normal myelin were often found but either contained no axon or were associated with an axon that was granulated. Degenerating myelin was similar to that already described, either homogenized with no period-

icity or in a loosely spiralled form. A few small normal myelinated fibers (0.5 um in diameter) were found with myelin structure similar to that in unoperated tissues.

Four Weeks

LIGHT MICROSCOPY - Vacuolization was no longer as apparent in the proximal segments as at earlier times, and it was obvious that the tissue was primarily of a peripheral nerve origin. Small fibers with no apparent myelin sheaths were observed traversing the connective tissue areas.

Within the graft area, small areas of extremely degenerated peripheral tissue were observed. The cytoplasm of phagocytic cells was vacuolated and filled with lipid particles, giving the tissue in this area a foamy appearance.

Distal to the graft, the optic nerve tissue did not appear to be different from that of earlier animals.

ELECTRON MICROSCOPY - In nerve segments both proximal to and within the graft itself, there was an absence of a CNS-type glial environment. All cellular elements were located in a peripheral-type environment which included abundant collagen and basal laminae surrounding neuronal and supporting elements. Myelin debris was always ap-

parent and was found within Schwann cell cytoplasm. Many unsheathed fibers were found with diameters from 0.8 to 1.2 um (figs. 38 and 40). These fibers were often surrounded by the same redundantly folded basal lamina seen in earlier animals. Occasionally, an unsheathed axon was seen to be intimately associated with a Schwann cell process (fig. 38). A few growth cones were found (fig. 41).

Occasionally, growing fibers were observed traversing the optic nerve - connective tissue interface (fig. 39).

Tissue distal to the graft was composed predominantly of CNS elements (fig. 42 and 43). An increased glycogen content was noted in the astrocytic processes. Degenerating optic nerve tissue was present in the extracellular spaces similar to that described in earlier animals. A few phagocytic cells containing many lipid inclusions were also found. Rarely, small myelinated fibers with diameters of up to 0.8 um were observed in the glial environment (fig. 43).

Six, Seven, and Ten Weeks

LIGHT MICROSCOPY - Myelinated fibers were observed in proximal, graft, and distal segments of nerve tissue in a connective tissue environment (figs. 44 and 45). These fibers were more obvious at seven and ten weeks

with more fibers enclosed by thicker layers of myelin.

ELECTRON MICROSCOPY - Groups of unmyelinated fibers (0.3 to 1.4 um in diameter) were present, ensheathed by Schwann cell cytoplasm (figs. 46 and 49). Numerous heavily myelinated axons (1.9 to 2.4 um in diameter) were also observed (figs. 47, 48 and 50). The myelin was characterized by 18 or more major dense lines and a 200 A periodicity. Mesaxons and lips of Schwann cell cytoplasm containing an occasional nucleus were sometimes found in association with these myelinated axons. These fibers were found in a peripheral nerve environment in all segments, with the fibers becoming more numerous and more mature from seven to ten w.p.o. (fig. 55).

Often, single sheathed axons or small groups of myelinated or unmyelinated fibers were found partially encircled by long thin cytoplasmic extensions forming a "tube" around the fibers and their associated Schwann cells (fig. 47). These cytoplasmic extensions were apparently of fibroblastic origin. They contained few inclusions or organelles, unlike Schwann cell processes which usually appear packed with ribosomal rosettes and other cytoplasmic organelles.

Scattered evidence of myelin debris (fig. 45) was still occasionally present within Schwann cell cyto-

plasm. CNS type tissues were no longer present in proximal, graft, or distal tissue segments.

Optic Nerve Transection Without Peripheral Grafting

TWO WEEKS - ELECTRON MICROSCOPY - Many unmyelinated and myelinating fibers of varying diameters (0.4 to 1.4 um) were seen in an optic nerve environment proximal to the transection (fig. 51). These fibers appeared to be undergoing regrowth and myelination similar to that described for the one-week postoperative grafted tissue.

Distal to the lesion, marked optic nerve degeneration was observed similar to that in the grafted animals (fig. 52). Fragmented fibers, rare phagocytic cells, and myelin debris without associated axons were often observed.

Glial limiting membranes were similar to those found in grafted animals. Axons were never found in the connective tissue areas adjacent to external and vascular limitng membranes.

EIGHT WEEEKS - ELECTRON MICROSCOPY - Nerve fibers other than those undergoing degeneration, were never found in any of the segments. Marked gliotic scarring and degeneration were obvious and similar to that observed in the distal segments of grafted optic nerve from three to four w.p.o. (fig. 54). The glial limiting membranes

which were found at any connective tissue-optic nerve interfaces, were similar to those described previously in grafted animals (fig. 53).

DISCUSSION

The first observation was that without peripheral nerve graft, the transected and sutured optic nerve of the rabbit appeared to undergo an abortive attempt at regeneration. This abortive regeneration was expected based on studies on transected mammalian spinal cords (Guth and Windle,'73). The apparently growing fibers present in the proximal stump of the optic nerve two weeks postoperatively (w.p.o.) were ultimately replaced with glial scar tissue by eight weeks.

With incorporation of a peripheral nerve graft between transected stumps of optic nerve, the initial regenerative attempt in the CNS appeared similar. However, rather than apparent degeneration of the growing fibers and their replacement by supporting elements, growth continued to occur in the peripheral nerve environment. Progression was observed from growth cones and less mature unmyelinated fibers (1 to 4 w.p.o.) to more mature myelinated fibers (6 to 10 w.p.o.).

Despite typical glial and connective tissue scarring, the growth of axons did not appear to be hampered and axons were found making their way through the tissues. In some instances, growing fibers were observed passing across the interface between the glial limiting membrane and the peripheral nerve, suggesting

that the limiting membrane may not be a potential barrier to elongating axons. It is not known why typical scarring did not prevent the growth of nerve fibers as has usually been reported in the literature. Hess ('56) also has concluded that glial scarring is not the reason underlying abortive regeneration in the CNS. Perhaps experiments should be reexamined for other answers as to why the regrowth of fibers is prevented.

The fact that these growing fibers were seen in central nervous tissue proximal to the graft at one w.p.o. and not in other segments (fig. 55) supports the hypothesis that these fibers are of probable retinal origin and are not sprouts growing in from adjacent peripheral nerves. The presence of similar fibers at two weeks in the non-grafted animal substantiates that the fibers could not be in any way from the graft itself or collateral sprouts from adjacent peripheral nerves utilizing the graft for entryway. Further, the fibers are probably not simply degenerating demyelinating optic nerve fibers since they initially are of such a small diameter relative to the fibers of the typical optic nerve population. Evidence in other CNS tissues indicate that sprouting peripheral nerves will not penetrate for more than a few millimeters into a glial tissue environment (Aguayo et al.,'79; Weinberg

and Spencer,'79).

Richardson et al. ('82a) reported abundant unmyelinated fibers two to four weeks after intracranial transection of the rat optic nerve. These were found 0.5 mm from the globe of the eye and were confirmed by autoradiography to be of retinal and not PNS origin. He also reported a retrograde axonal degeneration of those fibers so that only a few fibers remained 64 weeks after surgery. He proposed that this lack of sustained regrowth was due to retinal ganglion cell degeneration although he never examined the retina. Unpublished studies from this lab (Phillips,'81) indicate that normal appearing ganglion cells are present as late as 32 weeks after optic nerve transection in the rabbit. Preliminary light and electron microscopic examination of retinal tissues from this experiment also show that a viable population of ganglion cells are present as late as 10 w.p.o. Therefore, the rapid degeneration of the fibers in Richardsons' experiment is probably not due to a degeneration of the parent ganglion cells. It is of course possible that the growing fibers observed in this current study degenerate after 10 w.p.o., but it seems unlikely since at 10 w.p.o. there is no evidence of degeneration in the fiber population and small growing fibers are still abundant. In the same experiment,

Richardson et al. ('82a) also attempted to utilize a peripheral nerve graft by placing tibial or peroneal nerves adjacent to the intracranially transected optic nerve. He reported no obvious differences between grafted and ungrafted tissues using histological techniques. However, sutures or other methods were not used to maintain apposition to the optic nerve. Ultimately, many grafts were reported not to be in contact since transected ends of the optic nerve apparently pulled away from the graft as the nerve underwent necrosis. It is assumed that the more successful growth of fibers in the present study, when compared to Richardsons' work, may be related to the more consistent apposition of the graft and the optic nerve tissues. Also, the graft in the present study was placed in much closer proximity to the retinal cell sprouts. At a greater distance from the nerve sprouts, the probability that the peripheral nerve tissue will have a favorable effect on growing fibers may be negligible.

Unsheathed fibers were first seen amid astrocytic processes in the optic nerve segment close to the eye and the ganglion cell bodies. At later post-operative dates, these fibers were sucsessively observed closer to the graft, within the graft, and finally in the distal

segments of nerve (fig. 55). Growth cones were found along the advancing front. Ensheathment of these fibers by Schwann cells began in the more proximal segments at three to four w.p.o. and myelinated fibers were observed from five to ten w.p.o. Ultimately, normal-appearing, mature myelinated fibers were found throughout the nerve segments from six to ten w.p.o. In later animals, fields were found with many myelinated and unmyelinated fibers, both sheathed by cells of Schwann, apparently extending from the eye toward the brain (fig. 55). The observation that these fibers make their initial appearance in proximal and then in distal segments strongly argues against the supposition that they might be sprouts from adjacent autonomic or sensory nerves. Autonomic and sensory nerve fibers would grow from distal to proximal in the labelled segments since their cell bodies would generally be closer to the brain.

A substantial population of unmyelinated fibers as found in older animals, is unusual in optic nerve (Bruesch,'42). Since optic nerve fibers are normally of a smaller diameter (Vaney and Hughes,'76) than most sciatic nerve fibers and since many investigators feel that the signal for myelination is correlated with the increasing size of nerve fibers, then perhaps the Schwann cell only recognizes the larger growing fibers

as being ready for myelination. It may also be that over a longer period of time these axons would eventually be myelinated.

The sciatic nerve tissue appeared to invade the optic nerve tissue, totally replacing CNS tissue in the proximal segment by three to four w.p.o. and the distal segment by six w.p.o. It is interesting that this replacement was first observed proximally at the same time as the regrowth of fibers begins. As these fibers proceeded to grow distally the glial environment was simultaneously replaced by the peripheral nerve environment. Perhaps the fibers only continue to elongate into areas where the PNS tissues preceed them. Investigators who have experimented with peripheral grafts in the CNS have not reported this tissue replacement (Weinberg and Raine,'80) although Gledhill ('73) has stated that Schwann cells are capable of migrating into the CNS. Blakemore ('77), on the other hand, has reported that PNS tissues will not invade the neuropil of CNS tissues. Some interesting questions to explore would be exactly how much more of the CNS tissue the invading PNS tissue would replace over time, what the ultimate fate of these growing fibers would be, and if they would eventually make appropriate synaptic connections in the brain.

Previous experimentation has shown that Schwann cells in a donor PNS graft survive, multiply, and ensheath regenerating CNS axons (Aguayo et al.,'79; Blakemore,'77). Growing axons in PNS regeneration experiments characteristically are observed by four days post-operatively in the space between the Schwann cell and its' respective basal lamina (Nathanial and Pease,'63). Eventually, gutters form in the Schwann cell cytoplasm for embedment of the axons and ultimate myelination (Nathanial and Pease,'63). In this study the smallest axons were not usually found between the Schwann cell and its basal lamina but rather, they were found as isolated processes external to the Schwann cell basal lamina and surrounded by their own basal lamina. At four weeks, some of the fibers were found parallel to and in apposition to a Schwann cell cytoplasmic extension, oftentimes with separate basal laminae. Others were found embedded in grooves in Schwann cells. By six weeks, isolated axons were no longer found but the tissue characteristically contained numerous unmyelinated fibers. It is not known why the earliest fibers observed in this study were not found in association with Schwann cells as has been reported in other experiments. One possible explanation could be that these fibers are extremely small and

difficult to locate except at higher magnifications. Since one most often uses the lower magnifications when scanning tissues, these fibers could easily be missed. Another explanation might be that since these fibers are of retinal origin, and not regenerating PNS fibers, they do not normally associate with Schwann cells and thus grow into the spaces between these cells rather than immediately adjacent to them. After a period of growth and enlargement of these fibers, perhaps the Schwann cells then migrate toward the fibers and begin ensheathment as is observed by the third and fourth w.p.o.

Redundantly folded (fluffy) basal laminae were most often observed around isolated unsheathed axons in connective tissue three to four w.p.o. This highly folded basal lamina has been reported before in regenrating CNS tissues (Weinberg and Raine,'80). Nathanial ('63) has proposed that such laminae result from the collapse of the original neurilemmal contents and act as guides for the process of regeneration. If this were true then examples of this basal lamina should be easily found in degenerating peripheral nerve before the advent of the growing axons, yet this was never seen. Occasionally, redundantly folded basal lasminae were found associated with extensions of Schwann cell cytoplasm at three to four w.p.o. Since this experiment has only shown this

folded laminae associated with growing axons and cellular extensions, perhaps they are not merely remnants of the neurilemmal sheath but are indicative of protraction, retraction, and expansion of these mobile extensions.

From six to ten weeks after graft surgery, partial cytoplasmic tubes were found surrounding one or more Schwann cell units and their associated axons. These tubes were formed from thin cytoplasmic extensions which appeared to be of fibroblastic origin and probably represent the beginning of endoneurial tubes. Hall ('83) has reported similar findings in remyelinating peripheral nerve, but has considered the tubes to be supernumerary Schwann cell processes since axon sprouts were occasionally embedded in their cytoplasm. Only rarely were small (0.2 um) electron lucent structures found embedded in the cytoplasmic extensions in this experiment. Because of their size and lack of organelles they were not considered to be axons. Also, the long thin cytoplasmic extensions contained only a scanty amount of the organelles normally found abundantly in Schwann cells. Fibroblasts have often been reported be a spindle-shaped cell with long thin cytoplasmic extensions, containing few organelles and occasionally containing small lipid vacuoles (Bloom and

Fawcett,'75). These small electron lucent strucutres found in the cytoplasm of these "tubes" are most likely lipid vacuoles contained within fibroblasts.

The rate of optic nerve degeneration in rabbits appears to be much slower than that reported in lower vertebrates. Turner ('74) observed that optic nerve degeneration is virtually complete by fourteen days post-operative in the newt, with the glial cells playing a major role in phagocytizing and lysing the myelin debris. Microglia have been reported to phagocytize degenerating myelin and axonal debris following axonal lesions in the CNS of higher verterates (Fulcrand and Privat,'77). Kao ('77) identified many vacuolated microglia by light microscopy two weeks after spinal cord transection. Extreme vacuolization was also observed by light microscopy in the degenerating CNS tissues of this experiment. Although microglial-like cells were found using electron microscopy, minimal phagocytic activity was observed and evidence of tissue degeneration was still apparent in distal segments many weeks post-operatively. Much of this vacuolization seen by light microscopy was explained by the presence of numerous myelin "ghosts" (without axons) and spiral-led degenerated myelin seen under higher magnification in this study.

Normal appearing CNS axons were observed as late as four w.p.o. Brindley ('66) has reported histological evidence for the existance of normal fibers up to 337 days after transection of the optic nerve of the cat. These small nerve fibers observed in this study and by Brindley ('66) many weeks after nerve lesions, could indicate the presence of efferent fibers in the optic nerve. This statement is based on the observation that nerve segments closest to the cell body degenerate slower than severed segments (Peters et al.,'76). However, similar small axons were only rarely observed in control optic nerve, and since previous anatomical studies have not reported the existance of efferents in rabbits (Coleman et al.,'76; Vaney and Hughes,'76), it seems unlikely that they are present. In studying the rate of Wallerian degeneration in cat optic nerve following enucleation, Crevel ('63) found occasional fibers (less than 3u in diameter) using the light microscope at six w.p.o. By twelve w.p.o., the nerve was composed of mostly glial tissues. In this study, most fibers found in the distal segments of the experimental nerve from 1 to 4 w.p.o. were from 0.5 to 0.9 um in diameter. These are similar in size to the majority of fibers described by Crevel ('63). Therefore, these fibers are apparently not distal extensions of a slowly

degenerating efferent fiber population, but rather are remaining fibers of an extremely slow degeneration of retinal cell axons in the rabbit optic nerve.

Peripheral nerve, on the other hand, degenerated rapidly in this experiment and was actively phagocytized by Schwann cells. By six to ten w.p.o., degeneration was only occasionally apparent.

Large areas of tissue necrosis, cysts, or large vacuoles were never observed in the transected optic nerve in this experiment, as opposed to those reported in transected spinal cord (kao et al.,'77; Richardson et al.,'82). Blood supply to the area appeared adequate at all post-operative dates, perhaps due to an overlap of supply between nutrient arteries entering the nerve (McConnell,'64). Isolation of the small uniform fiber tract allowed a more precise determination of the morphological findings observed. Thus, the optic nerve was confirmed to be a simpler experimental model than the spinal cord for use in CNS regeneration studies.

Further experimentation should be done to further substantiate the evidence presented here for the CNS's capability to regenerate. There are several experimental approaches which could provide important answers. First, horse radish peroxidase studies could identify if the axons crossing the graft are

positively from retinal ganglion cells. Second, electrical recordings across the grafted area to establish if functional continuity of the axons occurs across the graft. Third, a silicon millipore chamber enclosing the graft and two edges of the transected optic nerve could provide better alignment of the stumps, allowing for a more positive identification of the original graft area, and allowing manipulation of the experimental area after the initial surgery. Fourth, retransection of the nerve, the use of Nauta stains, and examination of the lateral geniculate nucleus may determine if synaptic connections have been established. Fifth, retransection of the nerve and the proximal stump spliced into the lateral geniculate nucleus to observe if appropriate synaptic connections could be made by these axons with thalamic neurons and if they would continue to mature or would degenerate after failing to establish connections. Finally, the distal segments need to be studied closer to the brain ascertain 1) whether or not axons will cross the from the peripheral nerve environment into a glial environment or will continue to prefer the peripheral environment, 2) if the glial environment continues to be replaced by the peripheral nerve environment, and how far into the CNS tissue this replacement would ulti-

mately occur, 3) if the growing axons will continue to regenerate along the pathway of the old degenerating optic nerve and tract or will deviate into other adjacent tissues.

To summarize, interaction of the growing retinal nerve fibers with the peripheral nerve environment appears to produce the necessary milieu to allow continued elongation. It appears that by proper manipulation of nervous system elements, it may be possible for CNS fibers to regenerate across a transection.

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