side products of splicing. No such molecules have been identified so far; however, they may be generated only at low levels or may have a short half-life, thereby esçaping detection. Alternatively, a more complex signal (including, for example, specific secondary and tertiary structures) may be required to induce splicing.

The large introns of rabbit and mouse are almost as different as two random sequences. If the divergence were due to point mutations, the mutation rate within introns (0.4 to 1.5 $\times$ 10^{-8}) (*32*) would be at least 2 to 6 times higher than that of the β-globin silent sites (2.7 $\times$ 10^{-9}) or the variable regions of fibrinopeptides (2 to 4 $\times$ 10^{-9}) (*33*). An alternative explanation is that the internal part of the introns, whatever the genesis of introns may be, are subject to frequent or massive insertions and deletions; this would account not only for the unexpectedly strong sequence divergence of homologous introns, but also for the striking differences in their size. In the case of clearly related sequences (the 5′ flanking, 5′ noncoding, and coding—with the exception of replacement sites—mRNA sequence) the ratio of transitions to transversions is between 1.2 and 2.3. Comparison of two random sequences gives a value of 0.5, as would be expected statistically. We propose that, in eukaryotic DNA, as in the case of Qβ RNA (*34*), transitions are more frequent than transversions, but that selection at the protein (or RNA) level may lead to a modification of the ratio of transition to transversion. The finding that this ratio for the large introns is 0.63 could mean that the difference in sequence arises as a consequence of large insertions and deletions rather than multiple point mutations.

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Synaptic Regeneration in Identified Neurons of the Lamprey Spinal Cord

Abstract. *Identified reticulospinal neurons whose giant axons were severed after spinal cord transection were filled with horseradish peroxidase. Whole mounts and serial-section light and electron micrographs show axon regeneration across the spinal lesion and the formation of new synapses. Normal swimming activity returns in the spinally transected animals, although the regenerated synapses are in atypical regions of the spinal cord.*

Spinal transection in humans is considered to result in an irreversible loss of functions mediated by the damaged nerve fibers. Scattered reports of functional recovery after spinal cord injury have been imperfectly documented (*1*). In contrast, recovery of locomotor function after spinal transection has been reported in a number of the lower vertebrates: the tailed amphibians (*2*), teleost fish (*3*), and in the most primitive group—the cyclostomes, which include the lamprey (*4, 5*). The extent of structural regeneration in these lower forms is only partial, with the regenerating axons penetrating approximately 1 cm distal to the lesion, whereas in the normal cord they might have traveled for several additional centimeters. Rovainen (*5*) and Selzer (*6*) followed the course of the regenerating giant axons in larval lampreys through such a lesion with serial section light microscopy. They correlated functional recovery of locomotion with the growth (regeneration) of identified giant reticulospinal neurons (Müller and Mauthner cells) across the lesion for a distance of a few millimeters. They hypothesized that the functional recovery observed was probably due to synapses formed by the regenerating axons distal to the lesion, but Rovainen stated that "nothing is known regarding these newly established connections" (*7*).

We injected the marker enzyme horseradish peroxidase (HRP) into the identifiable giant reticulospinal neurons of the lamprey to examine the regeneration of axons and synaptic connections in the spinal cord. Serial sections studied with both light and electron microscopy give unequivocal ultrastructural evidence for the formation of new synaptic contacts by the identified regenerating spinal ax-

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ons in a region extending for several millimeters distal to the spinal lesion.

All experiments were performed on larval (ammocoete) sea lampreys (*Petromyzon marinus*). Stock animals were obtained from Maine and maintained at 15°C in tanks of well water provided with a substrate of fine sand. Animals 10 to 16 cm long were anesthetized in a solution of 0.3 g of tricaine (MS-222) per liter of well water. They were transferred to lamprey saline (*8*), and a 1-mm longitudinal incision was made in the dorsal midline between the second and third pharyngeal clefts. The exposed spinal cord was severed with a scalpel under direct visualization through a dissecting microscope. The incision was closed by gently squeezing the exposed muscle tissue together dorsal to the lesion, and the animals were returned to well water.

After the operations, only animals that began swimming again were taken for HRP injection. These animals were anesthetized, and the brain, together with 3 cm of nerve cord (including the lesion), was dissected out of the animal and maintained at 9°C. Tissue surrounding the lesion and most of the spinal cord was left intact to avoid damage to any regenerated fibers.

A 4 percent HRP solution in 0.05*M* tris buffer was iontophoretically injected into identified Müller and Mauthner cell bodies for 20 minutes in positive square-wave pulses of 0.5 second alternating with 0.5-second "off" periods. Preparations were maintained overnight at 4°C in oxygenated saline to facilitate diffusion of the marker enzyme.

The preparation was subsequently fixed in 2.5 percent glutaraldehyde in 0.1*M* sodium cacodylate buffer with 1 percent sucrose for 2 hours at 4°C, and washed in 0.1*M* sodium cacodylate with 2 percent sucrose for an additional 2 hours at 4°C. Treatment continued as follows: a 30-minute wash in 0.1*M* sodium borohydride in 0.2*M* tris buffer; 3 hours in 0.01 percent diaminobenzidine hydrochloride (DAB) in 0.2*M* tris buffer; 12 hours in DAB with H_2O_2 (two drops of 3 percent H_2O_2 per 10 ml of DAB); and 30 minutes in tris buffer.

For whole-mount examination, the brain and spinal cord were dehydrated and then cleared in methyl salicylate. The tissue may then be embedded in Spurr and thick-sectioned for light microscopy.

For electron microscopy, tissues treated with DAB were taken from the final wash and osmicated in 1 percent OsO_4 in 0.1*M* sodium cacodylate for 1 hour, dehydrated, and embedded in Spurr. Serial thick (5-μm) and thin (50-nm) sections were then cut. Thin sections for electron microscopy were stained with uranyl acetate and lead citrate.

Complete transection of the spinal cord in the lamprey resulted in immediate paralysis of the animal distal to the lesion as previously reported (*5*). Upon recovery from anesthesia, only spontaneous side to side movements of the head region anterior to the lesion occurred, and the animal could not propel itself over the substrate or through the water. At this time, tail pinching elicited rapid flexion of the body posterior to the lesion. Six lampreys that had regained the ability to swim 185 to 261 days after an initial spinal transection had new lesions made just proximal to the original transection. Paralysis distal to the second cut returned immediately. The first swimming was recorded in one animal 24 days after the second spinal cord lesion. Of the remaining five animals, two were able to swim again by 5 weeks, one died, and the remaining two showed no coordinated swimming activity even after 12 weeks, although they were both capable of some uncoordinated body undulations. Sham operations on a further four lampreys to expose the spinal cord rostral to an initial lesion did not prevent apparently normal swimming immediately upon recovery from the anesthetic.

Müller and Mauthner giant axons in the normal spinal cord are smooth and unbranched (*6*, *9*, *10*). Our results from 40 central nervous system (CNS) whole mounts in which the spinal cord was transected and injected with HRP confirm earlier findings that the injured giant axons may branch profusely during regeneration (*5*, *6*). These branches from the proximal portion of the parent axon initially grew distally toward the lesion. The incidence of branching was greatest just proximal to the lesion, but occasionally a branch was encountered in the brain. Some branches continued to grow across the lesion as far as 3.1 mm distal to the transection. Regenerating branches sometimes abruptly changed their direction of growth (Fig. 1). In Fig. 1 numerous dark giant axons from neurons filled with HRP are evident. The two most readily identifiable processes are the large axons of the two Mauthner cells situated on the extreme left and

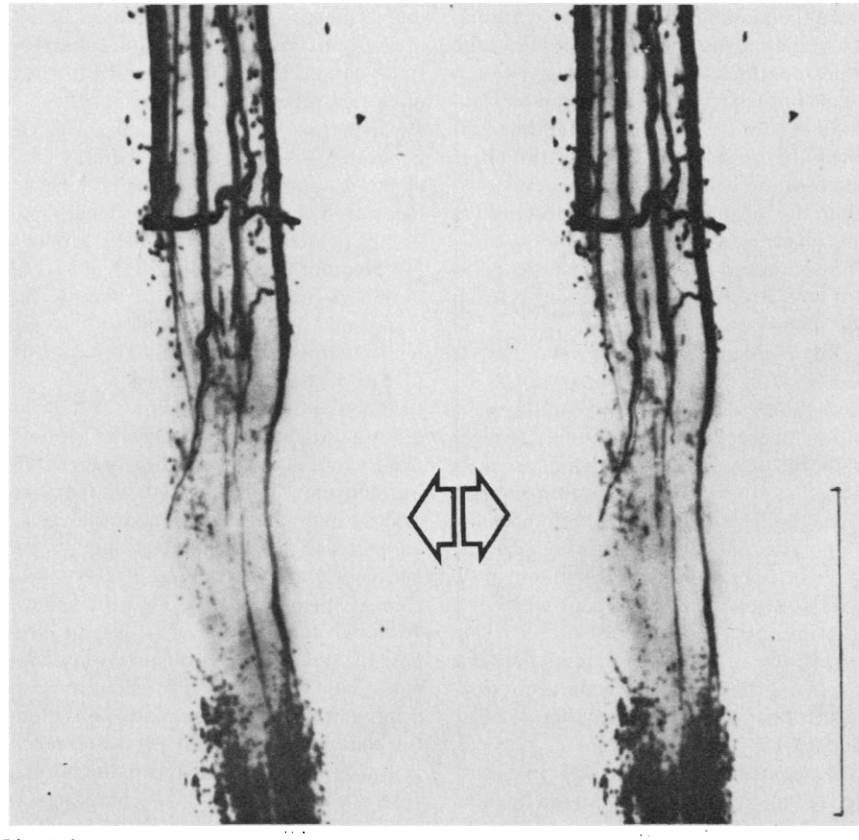

Fig. 1. Stereoscopic light micrographs of a lamprey spinal cord in the area of a complete spinal transection made 120 days previously. Whole-mount preparation; rostral is toward the top. Stained regenerating giant axons whose cell bodies were filled with HRP in the brain cross the lesion area (arrows). The filled giant axons branch both rostral and caudal to the lesion. The dark aggregate of blood cells tends to obscure the location and branching pattern of regenerated fibers caudally. Scale is 0.5 mm.

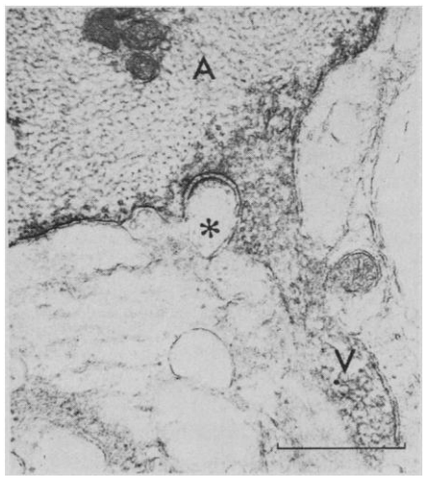

Fig. 2. Electron micrograph of part of an HRP-filled regenerated Müller cell axon 126 days after spinal cord transection. The HRP reaction product has accumulated along the plasma membrane. The section is 2075 μm distal to the transection point. It shows the filled fiber (*A*) from a bulbar cell making presynaptic contact with a small postsynaptic process (*) at the base of a sprout filled with lucent synaptic vesicles (*V*). Scale is 0.5 μm.

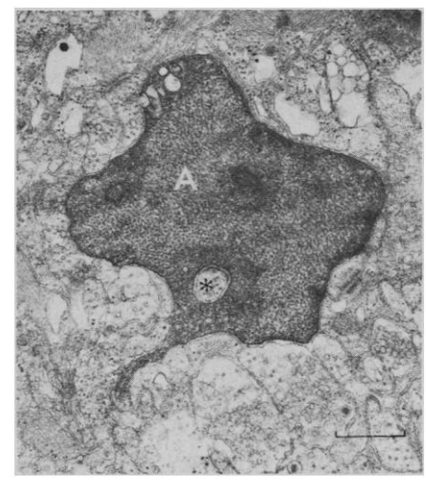

Fig. 3. Electron micrograph of an HRP-filled regenerated bulbar cell axon 126 days after spinal cord transection and 775 μm distal to the lesion. In this synapse, the postsynaptic process (*) has invaginated into the regenerated presynaptic axon (*A*) and is surrounded by lucent vesicles in the stained neurite. Note the defined synaptic cleft and the localized membrane densities. Scale is 0.5 μm.

right sides of the cord. The axon on the left stops short of the lesion, turns toward the midline, and branches. One major branch turns and grows rostrally; the other continues laterally and gives off a small branch, which grows caudally toward the lesion. The other Mauthner axon is the most pronounced of the fibers to cross the lesion. It bifurcates just distal to the lesion and becomes obscured in the black mass of invading blood cells. Other injected axons approach the lesion but only one other penetrates it to reach the distal cord.

The incidence of turning increased as regenerating axons approached the lesion. Some fibers turned rostrally while others proceeded on a relatively straight path through the lesion. One or more branches from a single neuron might cross the lesion while other branches belonging to the same cell could turn just anterior to or within the lesion and grow back toward the brain. Thus, whether a growing neurite penetrates the lesion seems due to local interactions between a growing tip and its immediate environment rather than a condition imposed on the cell as a whole.

Regenerating nerve axons that succeeded in crossing the lesion did not specifically follow their former tracts in the ventromedial region of the cord. Indeed, light and electron microscopy show no visible trace of the former tracts distal to the lesion in the area penetrated by the regenerated axons. Distal to the lesion, the regenerating fibers gradually grew toward the dorsal and lateral periphery of the cord.

We have correlated electron-opaque profiles of regenerated axons caudal to the lesion with thick sections serially traced from injected cell bodies in the brain. The HRP-filled axons show an overall increase in density rather than a granular deposit. The axoplasm contiguous with the axolemma was frequently more electron-opaque than the core of the fiber. The presence of translucent spherical vesicle clusters in the HRP-filled neurite, a uniform separation of membranes, and the paramembranous densities on the cytoplasmic surface of the apposed cell processes enabled us to identify such structures as synapses (Figs. 2 and 3).

In two preparations examined 126 and 141 days after lesions were made, we traced regenerating axons from 15 HRP-filled somata in the brain to a maximum distance of 3.1 mm beyond the lesion in the cord, at which point the HRP faded. We observed 39 synapses on these serially traced neurites in the two preparations, of which 36 were distal to the lesion. The few synapses observed proximal to the lesion relative to those distal is a result of concentrating our observations caudal to the lesion and probably does not reflect the relative number of synapses in these two regions. The regenerated neurites were invariably presynaptic, as judged by a complement of electron-lucent spherical vesicles 45 to 55 nm in diameter in the filled profiles. No pleomorphic vesicles were observed in any of the regenerated terminals, but occasional dense-core vesicles (70 to 170 nm in diameter) were encountered in six of the regenerated terminals. The uniform synaptic cleft measured 15.0 nm.

Branching from the normally smooth giant axons is characteristic of regenerating neurons. This induced tendency to branch extends to the synaptic level as an increase in the number and prominence of short lateral sprouts, which in some respects resemble dendritic spines. Although there is one ultrastructural report of Müller axon protrusions containing synaptic vesicles (*11*), they are not prominent in normal animals, and attention has been drawn to the smooth appearance of the giant axons in the normal cord (*9*). The sprouts of regenerating axons consist of a slender stalk 0.15 to 0.30 μm wide and as long as 1.9 μm, often terminating in a swollen knob. The sprouts, which are usually filled with lucent vesicles, frequently form the presynaptic element of a regenerating synapse. Synapses may form at the base of the sprout and also along its length. Two short sprouts from separate regenerating branches of the same bulbar cell (B_3) are shown in Figs. 2 and 3. A synaptic complex complete with membrane densities and a cleft is located at the base of the sprout in Fig. 2.

The two other regenerated synaptic conformations have been described in the normal preparation. In one type, the synapse occurs directly on the smooth shaft of the neurite and resembles en passant synapses. In the second, the postsynaptic dendrite invaginates into the HRP-filled presynaptic process (Fig. 3). Serial sections show that the invaginated fiber runs parallel to the axolemma. Two types of organelles, membrane densities and a few microtubules, are present in the postsynaptic dendrite.

The regenerated synaptic field distal to the lesion has features that differ significantly from the normal. In the regenerated synapses we observed, the giant axon elements were always presynaptic. In the normal cord, however, the giant axon may occasionally form the postsynaptic element of a synapse (*8*). Chemical synapses associated with the giant axons in the normal cord frequently have an adjacent gap junction with electrical coupling between the two neural elements (*9–12*). We have not observed gap junctions associated with any of the regenerated chemical synapses.

The reappearance of sinusoidal body movements leading to active swimming

suggests that the observed regenerated synapses are functional. This suggestion is further supported by the observation that a second lesion of the spinal cord just proximal to the first, in animals that had recovered the ability to swim, resulted in an immediate paralysis persisting for at least 24 days. The return of relatively normal locomotor function in the lamprey despite grossly aberrant patterns of regenerated synaptic connections supports similar findings in the goldfish (*13*). Despite the different spatial location of the regenerated junctions in the cord, the presynaptic elements may be still connecting to different regions of their normal postsynaptic target neurons.

The increase in branching may be related to the relatively short distance that regenerated neurites grow distal to the lesion. It has been hypothesized that neurons are genetically programmed to produce a relatively fixed surface area of plasma membrane (*14*). If this area is produced by profuse branching, the critical point at which growth stops may be reached closer to the lesion than if elongating growth were confined to a single neurite.

The formation of scar tissue (for example, ependymal or glial or both) has been implicated in producing a local physical barrier to the growth of nerve fibers across a lesion in the central nervous system (*15*). Scar tissue in the lamprey spinal cord appears to be composed principally of proliferating loosely aggregated ependymal cells. We found no collagen within the wound, although substantial quantities surround the normal cord (*16*). The resulting extracellular spaces between ependymal cells may provide channels for regenerating neurites to penetrate the lesion. A similar suggestion has been made for successful spinal regeneration in the newt (*17*).

We have demonstrated that axons from identified regenerating neurons grow through a spinal lesion and form new synapses caudal to the injury. This process is accompanied by functional recovery in the lamprey. The ability to follow growth and synaptic regeneration in identified reticulospinal neurons of the lamprey may permit this preparation to serve as a model for examining the cellular factors concerned with functional recovery of the injured vertebrate CNS.

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13 August 1979

Monoclonal Antibodies Defining Distinctive Human T Cell Surface Antigens

Abstract. *Three novel monoclonal antibodies (designated OKT1, OKT3, and OKT4) were generated against surface determinants of human peripheral T cells. Both OKT1 and OKT3 reacted with all human peripheral T cells and 5 to 10 percent of thymocytes but differed in their reactivities with T cell lines. By contrast, OKT4 reacted with 55 percent of human peripheral T cells and 80 percent of thymocytes. All three antibodies were selective for T cells in the peripheral blood in that they did not react with normal B cells, null cells, monocytes, or granulocytes.*

There is now ample evidence from work with mouse and man that lymphocytes, which appear relatively homogeneous morphologically, consist of subclasses of cells with differing functions and that these subclasses have distinctive cell surface antigens that can be identified with appropriate antiserums (*1*, *1a*). In man, such analyses of T cell surface antigens have been made with spontaneous autoantibodies, with heteroantiserums, and with serendipitously discovered markers such as rosetting of sheep erythrocytes (E) and bindings of the Fc (crystallizable fragment) of immunoglobulins (*2*). These techniques have provided valuable scientific insights but have been of limited value in clinical medicine because of the heterogeneity of antibody preparations obtained, frequent low titers, and the multiple absorptions required for production of specific heteroantiserums.

The hybridoma technique of Kohler and Milstein (*3*) provides a new approach to this problem whereby the multispecific responses to complex antigens are reduced to a series of monospecific responses by cloning. A number of hybridomas secreting monoclonal antibodies to various human cell surface antigens have been described (*3*, *4*). We report our methods for preparing monoclonal antibodies to the cell surface antigens of human T cells by immunizing mice, fusing their spleen cells with a mouse myeloma

Table 1. Immunofluorescent binding data from hybridoma supernatants in two fusion experiments. Single cell suspensions from mice immunized with human peripheral E^+ cells were mixed with P3 × 63Ag8U$_1$ myeloma cells and fused with PEG as described (*3*). After the fusion, cells were washed once in a mixture of DME, 10 percent IFS, and 10 percent IHS, and were resuspended in HAT medium (*5*, *12*). In experiment 1, cells were distributed in 1-ml portions into 100 wells, and in experiment 2, into 384 wells of Costar culture plates (Costar catalog No. 3524). Seven to 10 days after fusion, 0.5 ml of HAT medium (*12*) was added to each well. Supernatant from each well showing hybridoma growth was incubated with E^+ and E^- human mononuclear cells and binding was determined by an indirect immunofluorescence assay (*7*). In each experiment supernatants from only two wells showed specific reactivities for E^+ cells and these hybridomas were studied further.

Number of wells showing growth	Binding of supernatant with	
	E^+ cells	E^- cells
	Experiment 1	
50	−	Not tested
23	+	+
2	+	−
	Experiment 2	
277	−	Not tested
32	+	+
2	+	−

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