plexus, pineal body, and brainstem. Forebrains were homogenized in ten volumes of Earle's solution in Hepes buffer (*p*H 7.4) by a hand-held Potter-Elvehjem homogenizer (0.1-mm clearance; 30 up-and-down strokes). The homogenate was centrifuged at 1500*g* for 10 minutes; the pellet was resuspended in 0.25*M* sucrose and layered over a gradient (1.0 to 1.5*M* sucrose). The gradients were centrifuged at 58,000*g* for 45 minutes at 4°C in a Beckman SW 27 rotor. The microvessel pellet was transferred to a 153-μm nylon mesh, washed with cold saline, and either used directly or frozen and stored in liquid nitrogen until use.

15. The purity of our preparation was judged by the following criteria. (i) The absence of neuronal cells or glial cells, as determined by light microscopic examination of tissue stained with hematoxylin and eosin, phosphotungstic acid-hematoxylin (for glial fibers), elastin Von Gieson stain (for elastin fibers), or toluidine blue (for mast cells). Serial sections of this tissue also did not contain any of those cells or fibers. (ii) The absence of contaminating radioactivity from isotopically labeled synaptosomal or 1500*g* supernatant proteins (35*S*-methionine) when these fractions were added in significant amounts to untreated forebrain tissue just prior to microvessel preparation (I. Gozes and M. Moskowitz, unpublished observation). (iii) The absence of any measureable amount of prostaglandin D_2, the major prostaglandin in brain homogenates [M. Saeed Abdel-Halim, M. Hamberg, B. Sjoquist, E. Anggard, *Prostaglandins* **14**, 633 (1977)] in an incubation mixture containing brain microvessels (M. Moskowitz, unpublished observation). (iv) The presence of the putative transmitter, substance P, in amounts that are less than 0.1 percent of surrounding bovine brain tissue (D. Pettibone, S. E. Leeman, M. A. Moskowitz, unpublished observations).
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18. Rat brain microvessels (2 to 4 mg) were homogenized in 400 μl of 2*M* formic acid:ethanol (1:1), from which a portion was removed for protein determination. Duplicate 150-μl portions of a 35,000*g* supernatant were assayed by a modified version of a previously published assay [see Saavedra *et al.* (*16*)]. The reaction mixture was extracted into 3 ml of chloroform, washed with an equal volume of 1 m*M* HCl and dried with nitrogen gas. The residue was dissolved in 75 μl of absolute ethanol and applied to thin-layer chromatography plates, which were developed unidimensionally to a height of 10 cm, removed, and allowed to dry in a vertical position. This procedure was repeated eight times with pure chloroform as the solvent. Spots corresponding to authentic melatonin were scraped into scintillation vials, eluted with 250 μl of ethanol, and counted for tritium in 3 ml of toluene phosphor.
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Correct Axonal Regeneration After Target Cell Removal in the Central Nervous System of the Leech

Abstract. *The unique target neuron of a severed axon in the leech was selectively eliminated by intracellular injection of protease. In the absence of the target, the severed axon regenerated normally along its original pathway to the usual site of synapse, where it stopped growing without forming alternative connections.*

During development, the nervous system is assembled by the selective growth of its constituent neurons that contact certain target cells while rejecting others. In addition, many neurons in adults can regenerate severed axons to connect with their original targets, thereby restoring function to the nervous system. What role the target plays in axonal growth and in selectivity of connections is unclear. Interactions between growing neurons and their targets in the central nervous system (CNS) have been studied in populations of cells, often after disruptive surgical manipulations. Although conditions of growth during regeneration and development may not be identical, the similarities in the processes are striking.

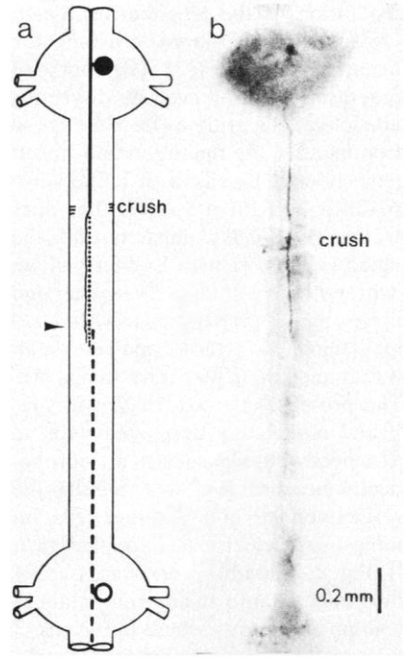

Fig. 1. Regenerating neuron in the ganglion at the top (anterior) in 1 month grows across the crush, along its severed distal stump [dotted line in (a)], and stops at the original synaptic region in the connective midpoint even when the target neuron [dashed line and open circle in (a)] has been selectively destroyed. (b) The regenerating neuron has been marked with intracellular injection of horseradish peroxidase 45 days after its axon was severed (*crush*) and the posterior S-cell (in lower ganglion) killed by an intracellular injection of protease. More extensive growth is not seen later. Arrowhead in (a) indicates the location of cross sections in Fig. 2.

Although the initial directed outgrowth of axons is apparently independent of either the target's location or its presence (*1*), the loss of synaptic targets can lead to the formation of aberrant synapses (*2*). However, recent studies of regenerating motor neurons in the frog have shown that extracellular cues are sufficient to direct the formation of synaptic terminals at the proper sites when the target muscle cells are removed without disturbing the surrounding environment (*3*). It would be useful, therefore, to remove the normal target of a single growing neuron in the CNS without disturbing its environs to determine which aspects of growth, if any, are affected. We now describe the regeneration of an identified axon in the CNS of the medicinal leech when its unique target neuron is selectively eliminated. In the absence of its target, this axon regenerates normally to its usual region of synapse without making aberrant connections.

The system of S-interneurons in the leech allows direct identification of regenerating and target cells. In each segmental ganglion there is one S-cell; it extends an axon both anteriorly and posteriorly halfway along the connectives, the axon bundles that link adjacent ganglia, to make an electrical synapse with the tip of the next S-cell axon (*4*). Fluorescent

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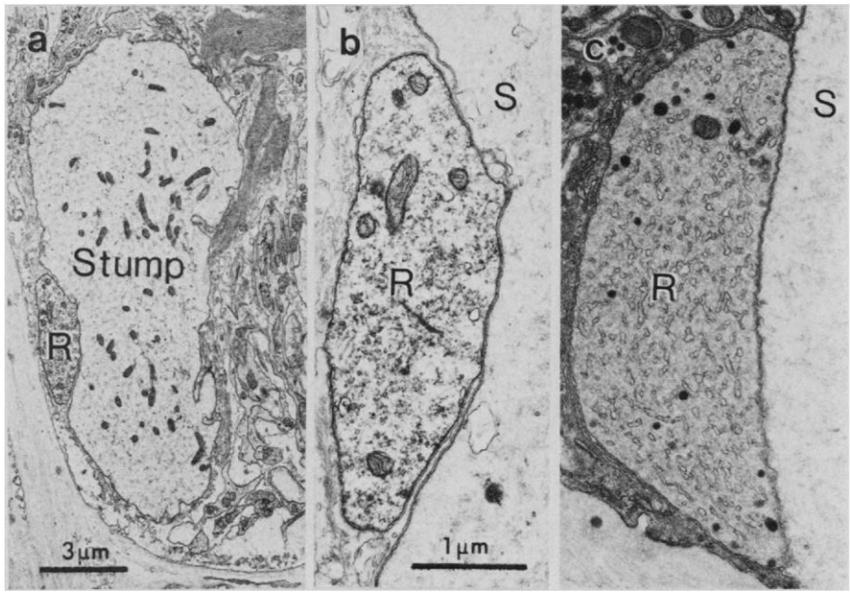

Fig. 2. (a) Six weeks after its target has been killed and its axon severed, the regenerating neuron (*R*) has grown to the normal region of synapse (arrowhead in Fig. 1a) near the end of its severed distal stump (*S*) where it has stopped. (b) The same section at higher magnification shows that in the axon tip (*R*) there is little of the smooth membranous reticulum found in growth cones at earlier times, such as that in (c) taken at 24 days. Same scale in (b) and (c).

dyes of low molecular weight such as Procion yellow (*5*) and Lucifer yellow (*6*) injected into one S-cell readily diffuse down its axons and selectively fill the adjacent S-cells (*7*), crossing into them at the electrical synapse. When the axon of one S-cell is severed, it regenerates along its distal axonal stump and forms a new electrical synapse with the axon tip of the adjacent S-cell. It takes about 1 month for the S-cell to grow halfway along the connectives. The new synapse, visible in the electron microscope, carries current from one S-cell to the next (*8*). Moreover, Lucifer yellow injected into the regenerated neuron passes into only the adjacent S-cells and fills no other axons (*9*). Thus, with this system of interneurons it has been possible to show that an individual regenerating axon forms an electrical synapse exclusively with its normal target at the original synaptic region.

When the S-cell axon is severed by cutting or crushing the connectives, the distal axon stump survives and remains electrically coupled to the target until the regenerating neuron itself contacts the target cell (*4*, *8*). Since metabolites can cross between electrically coupled cells (*10*), it seemed possible that maintained connection with the target could account for the stump's survival.

Because the S-cell axon can survive without a soma, removing the target cell body is insufficient to eliminate the target. Therefore, single S-cells were selectively removed from the nervous system of an otherwise intact leech by injecting the soma with a protease (*11*). To prompt regeneration of the neighboring S-cell, its axon was severed by crushing the connectives (Fig. 1a) (*12*). The course of regeneration was followed by dissecting chains of ganglia at times from 5 days to 5 months after the operation and injecting the injured S-cells with horseradish peroxidase (HRP), a marker that does not cross the S-cell synapse but fills the injected cell (*8*), or with Lucifer yellow, or with both. We studied 23 regenerated neurons in 18 preparations with electrophysiological methods, stained them, and examined them with microscopy (*8*).

The protease-injected S-cell was rapidly and completely destroyed with no direct electrophysiologically or morphologically measurable effects on either the severed axon stump or on other cells, including those electrically coupled to it (*13*) (Fig. 2, a and b). Moreover, Lucifer yellow injected into an adjacent intact S-cell stopped abruptly where the axons of the intact and protease-injected S-cells met.

When the target S-cell was killed, the adjacent injured axon regenerated across the crush and grew along its old distal stump in a manner indistinguishable from preparations (*8*) in which the target had not been killed. As in normal regeneration, the injured axon sprouted fine processes near its severed end. One or a few neurites grew across the crush, proceeded along the distal stump, reached the normal region of synapse in about 4 weeks, and then stopped growing (Figs. 1b and 2). Electron microscopy revealed that during the first 4 weeks of regeneration, the tips of growing axons were filled with a smooth membranous reticulum characteristic of growth cones (*14*) (Fig. 2c). After 6 weeks, the tips of regenerated axons had lost this reticulum and no longer appeared to be growing (Fig. 2, a and b). Axons were not seen to regenerate beyond the region of normal synapse or beyond the end of the severed distal stump for 5 months after surgery. In the course of normal regeneration, about 20 percent of injured S-cells failed to reach the target halfway along the connectives (*8*); in the present experiments, a similar percentage also failed to grow to that point.

The S-cells apparently did not make aberrant synapses. Impulses initiated in the regenerated S-cell, even after 2 months, failed to activate any other axons that could be recorded with a suction electrode applied to the connectives. Moreover, Lucifer yellow injected into regenerated S-cells did not pass into other axons in the connectives. When HRP and Lucifer yellow were injected together into the regenerated S-cells, the two markers were coextensive. Furthermore, electron microscopic studies of the region of synapse have revealed no close appositions between the regenerated axon and other cells, such as are seen at the normal electrical synapses (*8*); nor were chemical synapses seen. Together these findings demonstrate that when the target neuron was selectively eliminated, regenerating S-cells did not form other synapses like those made with the usual target.

We have no indication that S-cells that regenerated an axon toward a nonexistent target withdrew this unused axon, at least during the first 5 months. Nonetheless, we cannot be sure that regenerated axons survive without synaptic contact for longer times. There is reason to suspect they might retract (*8*).

The severed distal axonal stumps persisted as apparently healthy axons for as long as 5 months; thus, contact with an intact target cell was not required for their survival. Similar long-term persistence of isolated axon segments has been reported in a variety of animals (*8*, *15*). The mechanisms responsible for the prolonged survival of the distal stump are unknown; possibly nourishment is provided by the ensheathing glial cell (*16*). Under normal conditions of S-cell regeneration, the stump often served as a temporary synaptic target (*4*, *8*). We have seen Lucifer yellow pass from the regenerated S-cell into its distal stump,

which indicates that when the target cell was killed, the regenerating neuron could occasionally make an electrical synapse with its distal stump.

Since, when the target neuron is eliminated, S-interneurons regenerate to their normal region of synapse without making aberrant connections, something other than the target cell must be responsible for guiding the growing neuron and triggering it to stop growing. One likely candidate is the distal axonal stump, which generally survives during the month required for axonal regeneration, and often much longer. Regenerating neurons followed the distal stump apparently to its end and then stopped growing. The regenerating neurons may also be responding to other cues in the surrounding environment, left largely undisturbed by our surgical procedures. For example, either the ensheathing glial cell or extracellular components in the synaptic region remaining behind when the target S-cell is eliminated could be important. In the frog, when the target muscle cells are eliminated, end-plate specializations of the basal lamina provide cues to regenerating motor neurons (*3*), but neurons in the CNS of the leech do not have a basal lamina. What acts as a signpost to these growing neurons remains to be determined, but the target S-cell is not essential.

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Effect of β-Endorphin on Calcium Uptake in the Brain

Abstract. *The uptake of $^{45}Ca^{2+}$ by nerve-ending fractions from brains of mice was inhibited in vitro by 10^{-9}M concentrations of β-endorphin and in mice injected intraventricularly with 7 picomoles of β-endorphin. That the effect was a specific opiate agonist response of β-endorphin was demonstrated by use of the opiate antagonist, naloxone, which reversed the action. A role for β-endorphin in the regulation of calcium flux and neurotransmitter release should be considered.*

Ever since the discovery of peptides in the brain and pituitary that have opiate-like activity [the enkephalins (*1*) and β-endorphin (*2*)], attempts have been made to determine their physiologic role by comparing their pharmacologic effects with the effects of opiate drugs, usually morphine. Of the native peptides that have been isolated, β-endorphin appears most to resemble morphine with respect to its pharmacologic profile. β-Endorphin is active as an analgesic, being 20 to 30 times more potent than morphine when injected into the lateral ventricle and three to four times more potent when administered intravenously (*3*). Furthermore, sustained infusion of β-endorphin into the periaqueductal gray region, one of the most sensitive sites in the brain to opiates, produces opiate-like dependent behavior (*4*), and cross-tolerance between β-endorphin and morphine has been demonstrated (*5*).

Perhaps one of the most important effects of opiates is their ability to inhibit neurotransmitter release; this response may be related to changes in calcium ion flux. It has been demonstrated, for example, that morphine decreases acetylcholine (*6*) and norepinephrine release (*7*). Recently, the release-inhibiting effect of morphine on acetylcholine has been reported to be antagonized by calcium (*8*). Calcium also antagonizes the analgesic action of morphine and this effect can be enhanced by manipulations that increase brain membrane permeability to calcium and can be reversed by decreasing calcium availability (*9*). There is now evidence that a single dose of opiate effects a decrease in calcium content in nerve-ending fractions of brain homogenates (synaptosomes) (*10*), and that this decrease is dependent in part on reduced calcium binding (*11*) and uptake (*12*). In contrast, the changes in calcium disposition are reversed after sustained morphine administration, and the development of tolerance and physical dependence, that is, synaptosomal calcium uptake and binding, are increased (*10–12*). In view of these considerations, we deemed it important to study the effect of β-endorphin on synaptosomal calcium uptake.

Synaptosomes were prepared from homogenates of whole brains of male CD1 mice (21 to 25 g) according to a modified method described by Cotman and Matthews (*13*). Portions (1 ml) of a synaptosomal suspension were used for the determination of $^{45}Ca^{2+}$ uptake in the presence and absence of β-endorphin in vitro and in vivo. The suspensions were allowed to stand at 30°C for 2 minutes, then we added 1 ml of $^{45}Ca^{2+}$ [specific activity 0.05 mCi/mg in a solution containing 0.1 m*M* $CaCl_2$ (final concentration)], 3 m*M* $MgCl_2$, 3 m*M* adenosine triphosphate (ATP), disodium salt, and 50 m*M* tris buffer, *p*H 7.5. At fixed intervals thereafter (0.5, 1, 2, 4, 6, and 10 minutes), the Ca^{2+} uptake was terminated by separation of the synaptosomes from the incubation medium by rapid filtration through Millipore HAW (0.45 μm pore size) and washing three times with cold "stopping" solution (100 m*M* NaCl, 3 m*M* $MgCl_2$, 0.1 m*M* $CaCl_2$, and 50 m*M* tris, *p*H 7.5). The filters with the separated synaptosomes were transferred to glass counting vials containing 10 ml of scintillation solution (*14*), and the $^{45}Ca^{2+}$ present was determined by liquid scintillation spectrometry. Other portions of the synaptosomal suspension were used for the estimation of protein and calcium content. The amount of protein from synaptosomes was determined by the method of Lowry *et al.* (*15*) with bovine

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