Proc. Nat. Acad. Sci. USA
Vol 69, No. 3, pp. 636–639 March 1972

Regeneration and Changes in Synaptic Connections between Individual Nerve Cells in the Central Nervous System of the Leech

(segmental ganglia/motoneurons/sensory cell)

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Communicated by Stephen W. Kuffler, January 10, 1972

ABSTRACT **The central nervous system of the leech has been used for the study of the formation of new synaptic connections by regenerating neurons. In control leeches, individual nerve cells in adjacent ganglia are connected in an orderly and stereotyped manner, with only little variation. In the present experiments, a bundle of axons running between two of the segmental ganglia has been severed and allowed to regenerate. Subsequently, the axons reestablish synaptic connections between certain identified nerve cells in the adjacent ganglia, selectively and accurately. Thus, individual sensory cells in one ganglion show a high degree of neural specificity in reestablishing cell to cell connections with a motor cell in the next ganglion. The performance of the regenerated synapses, however, is significantly altered in a consistent manner. The normal balance between the effects of inhibitory and excitatory innervation in leeches with regenerated synapses is different from that seen in normal leeches, with marked overemphasis on inhibition. Similar alterations have also been seen in a series of ganglia at a distance from the site of the lesion. After the operation, therefore, a widespread modification of synapses occurs along the length of the nerve cord.**

Numerous experiments on invertebrates and vertebrates indicate that the synaptic connections of nerve cells are specific, in the sense that highly intricate connections are established between certain cells but not with others (1). At present, we have little information about the processes that enable nerve cells to find their targets either during development or regeneration, nor is it clear how reproducibly any one cell forms its connections in different animals. A different, but related problem concerns the stability of synapses: there is evidence that the effectiveness of synaptic action need not necessarily be fixed throughout the life of an animal but that it can be markedly altered, depending on the history of previous activity (2, 3). So far, experiments with the vertebrate nervous system have dealt with the reestablishment of synaptic connections between groups of nerve cells, rather than with regeneration of these connections between individual cells. The relatively simple nervous system of the leech offers a favorable preparation for studying in greater detail the specificity of synaptic connections. Individual sensory and motor nerve cells in different animals can be recognized, and their connections can be traced by recording electrically with intracellular microelectrodes (4, 5).

Earlier work has shown that axons in the central nervous system of the leech can regenerate after they have been transected, and that certain individual sensory cells can become reconnected to each other in a specific manner (6). These experiments also provided evidence that the performance of the animal's nervous system could be changed to compensate for the effects of the lesion. Thus, leeches moved in an obviously uncoordinated manner after the operation, but swam normally within a few weeks, even if regeneration had not yet occurred.

In the experiments reported here, it will be shown that connections between specific sensory and motor nerve cells are reestablished after regeneration of the nervous pathway between them; in addition, however, the regenerated synapses show certain consistent differences from the normal synapses with regard to the balance of excitation and inhibition. Similar changes also take place in the properties of synapses between nerve cells in segments of the nerve cord at some distance from the site of the experimental lesion.

METHODS

The procedures for operating on the leeches and tracing connections have been described in earlier papers (6, 7). Each of the segmental ganglia contains about 350 nerve cells. The ganglia are linked by two bundles of axons, the connectives, each of which contains many thousands of nerve fibers. Some of these axons run between the two ganglia and others extend over more than one segment. The leech is anesthetized with 8% alcohol, and one of the connectives is cut (see Fig. 1). The other connective is not severed, but it is often nicked by the incision to make certain that the cut has extended up to or beyond the midline of the nerve cord. This type of lesion abolishes the synaptic connections of the sensory cells upon the motor cells in the two adjacent ganglia on that side of the leech. Fibers do not cross from one connective to the other, and the sensory and motor cells that have been disconnected lie on the same side of the two ganglia as the cut connective. Through-fibers running to distant ganglia and to the brains at the two ends of the leech (8) are also interrupted by the operation.

Eleven regenerated preparations were used in this study. The leeches were examined for signs of regeneration and altered synaptic junctions from 45 to 260 days after the operation. Many of the leeches died within 1 month after the operation. In most of those that survived, the cut connective showed obvious signs of regeneration (see Fig. 2). Occasionally the connective did not grow back because of scar formation.

The method of tracing synaptic connections is to stimulate and record from individual, identified sensory and motor nerve cells (see below) with intracellular microelectrodes. One can conclude that connections exist between two cells if an impulse in one causes a synaptic potential in the other, but the observation of a synaptic potential does not on its own demonstrate that the pathway is direct.

The segmental ganglia that make up the ventral nerve cord of the leech survive well for many hours *in vitro* in physiologi-

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cal solutions containing: 115 mM NaCl, 4 mM KCl, 1.8 mM CaCl_2, 10 mM Tris–maleate (neutralized to pH 7.4), and 11.6 mM glucose. In the present experiments, 7.5 instead of 1.8 mM CaCl_2 was used routinely; at this concentration of CaCl_2 the synaptic potentials are increased in amplitude and are easier to discern.

RESULTS

Normal and regenerated connections of sensory and motor cells in adjacent ganglia

The sensory and motor cells used for studying regeneration can be reliably identified in any of the segmental ganglia that make up the ventral nerve cord by several physiological and anatomical criteria (4). Each of the sensory cells labeled *P* (pressure) or *N* (nociceptive) in Figs. 1 and 2 responds characteristically to mechanical stimulation of a well-defined area of the skin. They all make direct synaptic connections with a pair of large motoneurons (*L*) in the same ganglion, that in turn innervate longitudinal muscles (5). These connections mediate a segmental shortening reflex (7). An impulse in a pressure or a nociceptive cell also evokes excitatory synaptic potentials in the large motoneurons in the adjacent ganglia (Jansen, Nicholls, and Purves, unpublished data). An example of such interactions in a normal leech is illustrated in Fig. 1, which shows the excitatory synaptic potential of a motor cell (*L*) after an impulse in an *N* sensory cell of the neighboring posterior ganglion. In the normal leech, the synaptic potentials were usually similar whether the sensory cell stimulated was in the anterior or the posterior of the two neighboring ganglia (but, see below).

In leeches with a regenerated connective, synaptic connections were reformed between sensory cells in one ganglion and the *L* motor cell in the next ganglion. Examples are shown in Fig. 2. The properties of the regenerated synapses were, however, consistently different from those seen in control leeches with respect to the balance of excitation and inhibition. In one direction of stimulation, the response resembled the normal. Thus, when a sensory cell in the anterior ganglion of the pair was stimulated, an excitatory, depolarizing synaptic potential was recorded in the *L* motor cell in the posterior ganglion. On the other hand, Fig. 2*B* shows that when the direction of stimulation was reversed (posterior sensory → anterior motor), an inhibitory, hyperpolarizing synaptic potential appeared in the *L* motor cell. (In this experiment, the normal uncut connective was severed just before testing for regeneration, to make certain that impulses could travel between ganglia only by way of regenerated nerve fibers.)

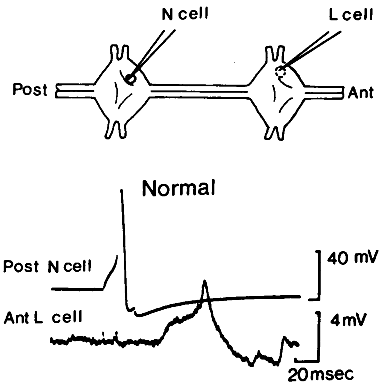

FIG. 1. Diagram to illustrate a sensory cell responding to noxious (*N*) stimulation of the skin, and the large longitudinal motoneuron (*L*) that innervates the longitudinal muscles. The *L* motor cell lies on the deep, dorsal surface of the ganglion and its outline is dotted (see Fig. 4). Each of these cells can be unambiguously identified in any of the segmental ganglia. The ganglia are linked by two bundles of axons, the connectives. When one connective is cut, the sensory and motor cells on that side are immediately disconnected. The traces are intracellular recordings from a nociceptive sensory cell (*upper trace*) in one ganglion and the *L* motor cell in the adjacent anterior (Ant.) ganglion. An impulse in the *N* cell, produced by a current pulse, leads to an excitatory synaptic potential in the *L* cell. Similar potentials are seen after an impulse in the pressure sensory cells (*P*; Figs. 2–4) and also when the direction of stimulation is reversed (anterior sensory → posterior (Post.) motor).

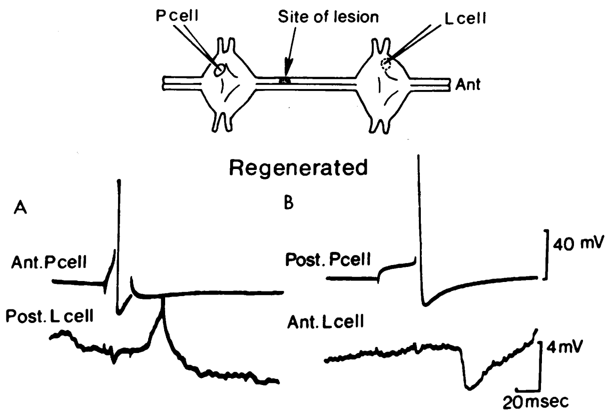

FIG. 2. Synaptic potentials in *L* motor cells in regenerated preparations. The diagram above shows the site of the lesion in one of the connectives linking two ganglia. Record *A* shows the response in a regenerated preparation that had been operated upon 70 days beforehand. The *upper trace* is from a sensory *P* cell in the anterior (Ant.) ganglion; the *lower trace* is from an *L* motor cell in the posterior (Post.) ganglion. The depolarizing synaptic potential recorded in the *L* cell is similar to that observed normally. Both of these cells were on the side of the original lesion. Record *B* shows the hyperpolarizing response in an *L* cell in a regenerated preparation after stimulation of a *P* cell in the adjacent posterior ganglion. In this preparation, the connective had been cut 116 days beforehand. Inhibitory potentials were always seen in the anterior *L* cell after regeneration when the direction of stimulation was from a sensory cell in the posterior ganglion.

This asymmetry in the synaptic effects was found invariably whenever synaptic connections had been reestablished. All 11 leeches examined from 45 to 260 days after section of the connective showed the inhibitory synaptic connection between the sensory cells of the posterior ganglion and the *L* motor cell of the anterior ganglion. In some of these preparations, the normal excitatory component reappeared, but it was smaller and, therefore, was obscured by the large inhibitory potential. Regenerated connections from sensory cells in the anterior ganglion to the *L* motor cell in the posterior ganglion were found less often; when they did occur, in 4 out of 11 leeches, the synaptic potentials appeared to be normal, as in Fig. 2*A*, and were purely excitatory.

Altered synaptic connections between ganglia at a distance from the lesion

At first it appeared as though the inhibitory component observed after regeneration might represent some form of error

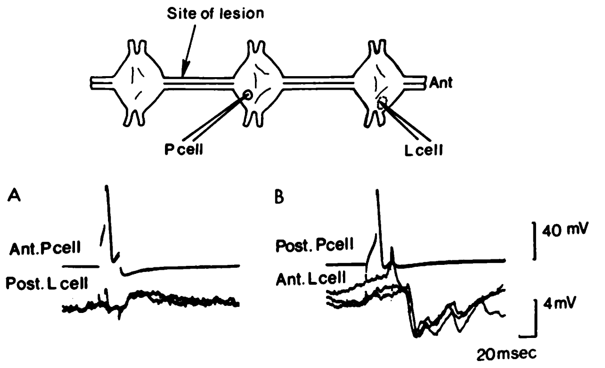

FIG. 3. Altered synaptic connections between sensory and motor cells in a pair of ganglia distant from the lesion. The leech had been operated on 125 days earlier. The records are taken from a pair of ganglia one segment away from the site of the cut connective. An impulse in the anterior (Ant.) sensory cell evokes a normal depolarizing synaptic potential in the *L* motor cell in the adjacent posterior (Post.) ganglion (*A-superimposed traces*). In the opposite direction (*B-superimposed traces*), stimulation of the posterior *P* cell causes an inhibitory potential similar to that seen over the regenerated connective of the same leech. Other ganglia in the nerve cord showed similar changes on both sides of the leech.

in reconnection. Quite unexpectedly, however, we found that similar changes occurred in connections between sensory and motor cells in adjacent ganglia along the length of the leech. The interconnections of these ganglia had not been directly severed by the operation, but presumably much of their normal input had been chronically cut-off, owing to the interruption of through-fibers. Fig. 3 shows examples of altered interactions between sensory *P* cells and the large *L* motoneurons in two ganglia taken from a leech that had been operated on 125 days previously. The connective linking this pair of ganglia was one segment away from the site of the lesion. Instead of the usual two-way excitatory effect, inhibition predominates in the direction posterior-sensory to anterior-motor, and the normal excitation is seen in the opposite direction.

We have found such changes in synaptic connections between ganglia in eight leeches examined with survival times from 70 to 260 days after the operation. We have not yet been able to define the earliest date for the changes to appear. The connections between ganglia were altered along the length of the nerve cord, in front of and behind the site of the lesion. For example, in one experiment in which a connective had been cut, we tested connections between sensory and motor cells in three pairs of ganglia situated in segments above and below the lesion. In each case, stimulation of the sensory cells in the posterior ganglion of the pair led to a predominantly hyperpolarizing response in the *L* motor cell of the anterior ganglion. Furthermore, the changes were present on both sides of the leech.

The similarity between the alterations in the synaptic connections of pairs of neurons in distant ganglia and those found at regenerated synapses prompted a reexamination of the connections in the normal leech. When repeated synaptic responses were averaged on a computer of average transients or by photography, a small but consistent hyperpolarization of short latency became apparent in the connection from sensory cells to *L* cells in the neighboring anterior ganglion. In the opposite direction, however, the connections from anterior sensory cells to the *L* cells of the posterior ganglion always appeared to be purely excitatory. Although in normal preparations, the inhibitory connection to the anterior *L* cell was small and often not detectable, its existence does suggest that the marked changes in the synaptic connections after the operation represent an enhancement of a normal, but usually occult, synaptic pathway, rather than the formation of a new, abnormal pathway. It therefore seems that the balance between the normal combined excitation and inhibition of the motor neuron has been upset.

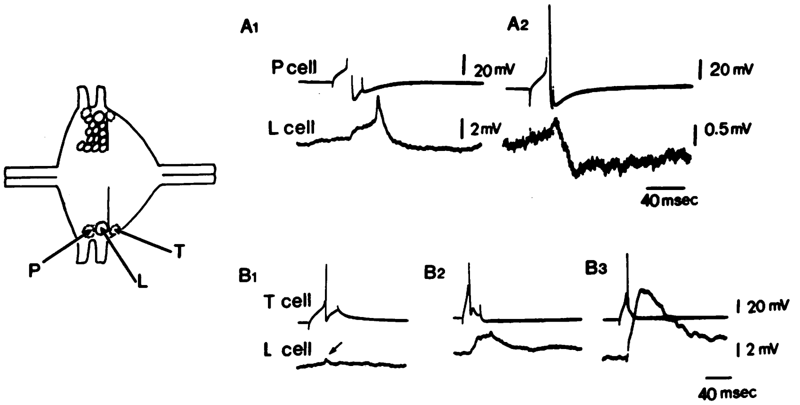

FIG. 4. Altered synaptic connections between sensory and motor cells within an isolated ganglion, in which all connections to the rest of the Central Nervous System and the body had been severed 37 days earlier (A_2, B_2, and B_3). The *diagram* represents the dorsal surface of the ganglion and indicates the position of the *T* and *P* sensory cells (*dotted outlines*) that lie on the ventral surface. The cells, in the vicinity of the *L* cell,that have been used to test for specificity (see *text*) are outlined in the upper part of the diagram.

The normal depolarizing synaptic potential of the *L* cell after an impulse in a *P* sensory cell is shown in A_1. In the isolated ganglion, A_2, an impulse in a *P* cell gives rise to a hyperpolarizing synaptic potential in the *L* cell. B_1 shows the normal interactions between a sensory cell that responds to touch of the skin (*T* cell) and the *L* cell in a control ganglion. A small but consistent synaptic potential (indicated by the *arrow*) is mediated through an electrical synapse (see text). In the isolated ganglion, B_2, the synaptic potential in the *L* cell after an impulse in the *T* cell is larger and longer lasting. Its magnitude is markedly increased when the *L* cell is hyperpolarized by steady current passed through the electrode (B_3). This indicates the presence of a chemical synaptic connection that cannot be discerned in normal ganglia.

Specificity of neuronal connections

In the normal leech, the connections from the *P* and *N* cells to *L* cells in neighboring ganglia are highly selective: the *L* motor cell is not just one of numerous indiscriminate targets for sensory fibers. After regeneration had occurred, the connections between sensory cells and the *L* motor cells were also specific but to a lesser extent, in the sense that synaptic potentials sometimes appeared in other cells where they are not normally observed.

To substantiate this, we activated *P* or *N* cells in a ganglion while recording from 10 or more neurons in the vicinity of the *L* motor cell in the neighboring ganglion. These cells, illustrated in the diagram of Fig. 4, make up a constant group, but an individual cell cannot yet be reliably identified on the basis of its size or position (4). In four normal leeches, the usual result was that synaptic potentials were observed in the *L* cell but not in any of these other cells. Occasionally, one neuron close to the *L* cell might also show synaptic potentials. In regenerated pairs of ganglia, we found a higher incidence of synaptic activity in these other neurons after an impulse in a *P* or an *N* cell in the adjacent ganglion. In most preparations one or two additional cells gave synaptic potentials, but it was not rare to find that only the *L* cell responded to an impulse in a sensory *P* or *N* cell as in the normal. In one preparation that had regenerated, we found evoked excitatory synaptic potentials or inhibitory synaptic potentials in four additional neurons, as well as a synaptic potential in the *L* motor cell.

In leeches with one connective cut, the selectivity of synaptic coupling also appeared to be reduced in altered regions of the nervous system; synaptic potentials could be recorded in cells other than the *L* motor cell more frequently than normal, in segments at a distance from the lesion.

Completely isolated ganglia

The appearance of changes at a distance from the lesion suggested that the synaptic interactions between cells might have been modified as a result of partial isolation of neurons from their normal inputs. Accordingly, we tested the effects of isolating a ganglion completely from the body and from the rest of the central nervous system by cutting all the peripheral roots and connectives. This leaves the ganglion in "organ culture" within the animal. In two such experiments there were marked changes in the connections of *P* cells to the *L* cell within the same ganglion. An example is given in Fig. 4. Fig. $4A_1$ shows the normal depolarizing potentials that are chemically and electrically mediated (7). In the isolated ganglion, the electrical synapse between *P* and *L* cells was still present, but a large hyperpolarizing inhibitory potential had supplanted the usual chemical excitatory potential (Fig. $4A_2$). The connections of touch cells onto the *L* cells were also altered in these isolated ganglia. In normal ganglia, the touch cells are electrically coupled to the *L* motoneuron by a rectifying junction, with no obvious sign of chemical transmission (7). Figs. $4B_1$ and $4B_2$ show that, in addition to the usual electrical synapse, a chemical synaptic component has become apparent in the isolated ganglion. The excitatory synaptic potential is greatly amplified by hyperpolarization of the postsynaptic *L* cell, a characteristic feature of chemical synaptic transmission. Thus, more complete section of the connections of a ganglion from the rest of the animal leads to changes not only between ganglia but also in the synaptic organization within a ganglion.

DISCUSSION

The results confirm that regeneration can occur between individual nerve cells in the central nervous system of a leech with a high degree of specificity (6). The sensory cells become reconnected to motor cells in a consistent and selective manner, with few connections to other neurons. Although the appropriate cells interact once again after regeneration, it remains to be determined whether the pathway is direct. For example, inhibitory potentials are seen when a posterior sensory cell is stimulated in regenerated, altered, and, occasionally, in normal leeches. Electrical recordings do not indicate whether the same inhibitory interneuron is involved or whether new pathways are used after the operation. We have as yet no knowledge of the mechanisms that enable individual cells to find their targets, but as a working hypothesis we may assume that the same mechanisms that determine the formation of specific synaptic connections during embryogenesis are still active in the adult leech and guide the reestablishment of synaptic connections during regeneration.

The regenerated connections are in some respects different from normal, but the changes appear to be part of a process extending throughout the length of the leech. These changes, found throughout the nerve cord of the operated leeches, are equally interesting. One can imagine that the cause of the changed efficiency of the synaptic connections is either an alteration in the normal signalling, or the degeneration of through-fibers extending over the greater part of the nerve cord. It is tempting to speculate that the lesion causes large numbers of fibers that normally synapse upon the *L* motoneurons to drop out or become ineffective. This partial denervation could conceivably lead to chemical supersensitivity, to accessory sprouting of collaterals, and to the formation of new synaptic contacts; at present, one cannot distinguish between these and other possibilities. The results presented here do show that leech neurons can regenerate with a high degree of accuracy and that synapses within the cord are capable of undergoing marked changes in effectiveness.

We thank Dr. Dale Purves for many helpful comments and discussions and Miss D. Bosler for help with the photography. The work was supported by USPHS grant 5R01 NS 08277-03 and by a grant to J. K. J. from the National Multiple Sclerosis Society.

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