Functional regeneration following spinal transection demonstrated in the isolated spinal cord of the larval sea lamprey

(central pattern generator/locomotion)

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ABSTRACT   Axons in the larval sea lamprey can regenerate across the site of a spinal cord transection and form functioning synapses with some of their normal target neurons. The animals recover normal-appearing locomotion, but whether the regenerating axons and their synaptic connections are capable of playing a functional role during this behavior is unknown. To test this, "fictive" swimming was induced in the isolated spinal cord by the addition of D-glutamate to the bathing solution. Ventral root discharges of segments above and below a healed transection showed a high degree of phase-locking. This strongly suggests that the behavioral recovery is mediated by regenerated functional synaptic connections subserving intersegmental coordination of the central pattern generator for locomotion.

The larval sea lamprey is a useful model in studies of regeneration within the vertebrate central nervous system. Motor and sensory functions return 6–8 weeks after complete spinal cord transection (1–3), and this behavioral recovery is immediately eliminated upon retransection (2). Stimulation of the head can elicit reflexive curling (3) and sinusoidal swimming movements (4) in the tail. Coordinated electromyographic bursts have been observed in ammocoetes between segments above and below a healed transection (5). Spinal axons regenerate for short distances beyond the scar (1–3, 6), and new synapse formation has been demonstrated both physiologically (7) and morphologically (8). Despite these findings, it has still not been established whether the regenerated circuitry itself is useful in producing coordinated behavior. We now have found, by studying "fictive locomotion" in the isolated spinal cord, that the regenerated neural tissue can coordinate the swimming motor pattern across the healed transection site. Thus, the swimming observed in recovered animals results from functional regeneration of the intersegmental coordinating system of the central pattern generator (CPG) for locomotion.

MATERIALS AND METHODS

Experiments were performed on *Petromyzon marinus* ammocoetes 10–14 cm long (larvae 4–5 years old). The three control and eight experimental animals were chosen from the same pool of animals, all of whom were roughly the same size and therefore in the same stage of development. Eight larvae received a spinal transection under Tricaine anesthesia (3) at a location midway between the last gill slit and cloaca; all such animals lost coordinated swimming patterns the day of transection; seven of the eight were swimming normally when tested 2–6 months later. At that time the spinal cords were removed (9), cut into pieces 60–80 segments long (the healed scar was located in segments 30–40) and placed in a

10°C bath perfused with 100 mM NaCl/2.1 mM KCl/2.6 mM CaCl$_2$/1.8 mM MgCl$_2$/12.0 mM NaHCO$_3$/3 mM glucose through which 98% O$_2$/2% CO$_2$ had been bubbled. The appearance of the healed scar (Fig. 1) indicated a complete transection in all eight of these animals. Spinal cord pieces of comparable location from three ammocoetes that had not been given transections served as controls.

Bipolar suction electrodes, placed directly onto ventral roots (VRs) of segments rostral and caudal to the transection site, monitored motor output induced by addition of D-glutamate (0.25–1.00 mM) to the perfusate (9, 10). The signals were recorded conventionally and stored on a high frequency FM tape recorder. The bursting was replayed onto a chart recorder to produce a hard copy for analysis. At this stage, visualization of the temporal pattern of the activity was facilitated by rectifying the signals. A digitizing tablet and microcomputer were used to calculate and graph cycle periods and phase delays. Cycle period is defined as the time from one burst onset to the next burst onset in the same root. Phase delay is defined as the time between onset of discharges in a rostral and a more caudal VR, as a fraction of the cycle period of the more rostral VR.

RESULTS

VR Burst Patterns in Control Spinal Cords. Fictive locomotion in adult lampreys is characterized by a fixed phase delay of roughly 1% of the period per segment regardless of the frequency of bursts (9). This motor output pattern has been shown to be the *in vitro* analog of swimming in the intact lamprey (11). The VR discharge pattern in control larvae differed from that previously reported for adults. Upon addition of D-glutamate (0.25–1.0 mM) to the bath, the spinal cords first displayed a slow and often variable pattern of VR bursting with a mean period of 8.88 sec (13.80, 5.69, and 7.16 sec in the three cords; Fig. 2). These slow rhythms persisted for no more than 30–95 cycles. The phase delays between segments were longer and more variable than those seen during adult fictive swimming. Phase-delay histograms revealed preferred phases where the average of the median value was 3% (2%, 3%, and 4%) per body segment (Fig. 3). VR output became disorganized after the slow bursting (in the same D-glutamate concentration), until a more stable and faster rhythm emerged (Fig. 2). These faster rhythms had a mean cycle duration of 1.52 sec (0.80, 2.25, and 1.51 sec in the three cords) and an average median intersegmental phase delay of 0.67% (0.5%, 1.0%, and 0.5%) per body segment (Fig. 3). The two observed patterns of firing (slow and fast) may represent different movement patterns (e.g., burrowing and free swimming), although differences in phase delay for different movements have not as yet been observed (12).

Abbreviations: CPG, central pattern generator; VR, ventral root.
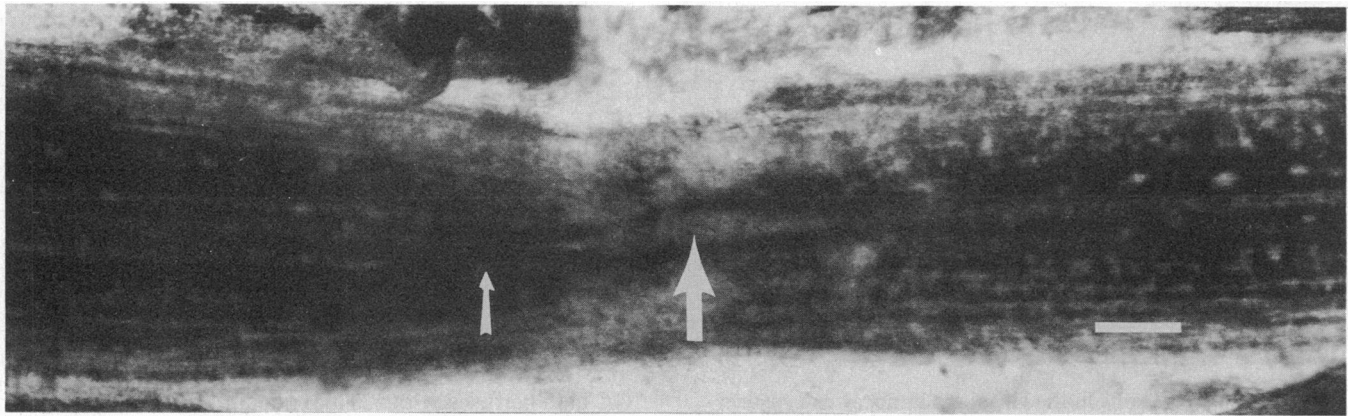†To whom reprint requests should be addressed.

FIG. 1.   Photomicrograph of the living spinal cord in the region of the healed scar from a previously transected ammocoete. Notice the discontinuity of the large axons (small arrow points to a proximal axon or bundle of a few Müller axons), the widening of the central canal (large arrow), and the narrowing of the cord in the region of the scar. Rostral is left. (Bar = 200 μm.)

VR Burst Patterns in Previously Transected Spinal Cords.
Simultaneous recordings from VRs above and below a healed transection showed evidence for coupled slow and/or fast bursting in all eight animals. In these animals, as in controls, addition of D-glutamate was followed by slow bursting, but patterns were stable enough to analyze in only three of the eight cases. These had a mean period of 7.66 sec (12.59, 3.74, and 6.65 sec; e.g., Fig. 4). In two animals, the preferred phase delays between VRs rostral and caudal to the scar [$\approx$3% (Fig. 5 *Top*) and 4% per segment] were similar to those in control larvae. In the third, it was considerably shorter (0.3% per segment). The distribution of phase lags across bins of 0.05 each (see Fig. 4 *Top*) was compared to random using a χ^2 test. The distributions of phase delays for all three slow patterns were significantly different from random ($P < 0.005$), despite a secondary cluster around zero in all three. The reason for the secondary cluster is unclear, but it was also seen on occasion in control spinal cords.

After lapsing into disorganized VR activity, the full 60- to 80-segment spinal cord pieces of the treated animals, unlike controls (Fig. 2 *Bottom*), did not develop coordinated fast bursting. However, removing equal numbers of segments from the rostral and caudal ends (with the new pieces 20–60 segments long) resulted in stable fast bursting (Fig. 4 *Bottom*) with periods of 0.72 $\pm$ 0.19 sec (mean $\pm$ SD, $n = 7$; see *Discussion* for the motivation for this procedure). The faster rhythms were coupled across the healed transection, as

judged by the uniform phase lags (Fig. 5 *Middle*), in seven of eight spinal cords. In five cases, including the one illustrated in Figs. 4 and 5, the phases hovered near zero, (i.e., synchronous bursts), which is slightly shorter than in controls. In two cords, the phase lags were near 3% per segment, which was considerably longer than in controls. The reason for this apparently bimodal distribution is not known. In the eighth cord, the phase lags were not constant.

When the scars were retransected, both rostral and caudal pieces continued to burst, but independently. Comparison of 100–200 cycles before and after retransection was used to confirm that the observed coordination was not the consequence of the rostral and caudal segments coincidentally bursting at similar but uncoupled frequencies. In seven of the eight spinal cords, the phase histograms were substantially different from those of the respective retransected cords (Fig. 5 *Bottom*). Although the phase delays before retransection varied more than in controls, the distributions of the phases between VRs rostral and caudal to the scar in the seven recovered animals were clearly not random [$P < 0.005$ (χ^2 test) in all cords], whereas after retransection they were ($P = 0.11$–0.75). In the eighth cord, from the animal that had not recovered normal swimming coordination, the VR patterns did not differ significantly before and after retransection. This last spinal cord exhibited good coordination during the slow rhythm. Therefore, all experimental ammocoetes clearly exhibited some degree of coordination across the transection site.
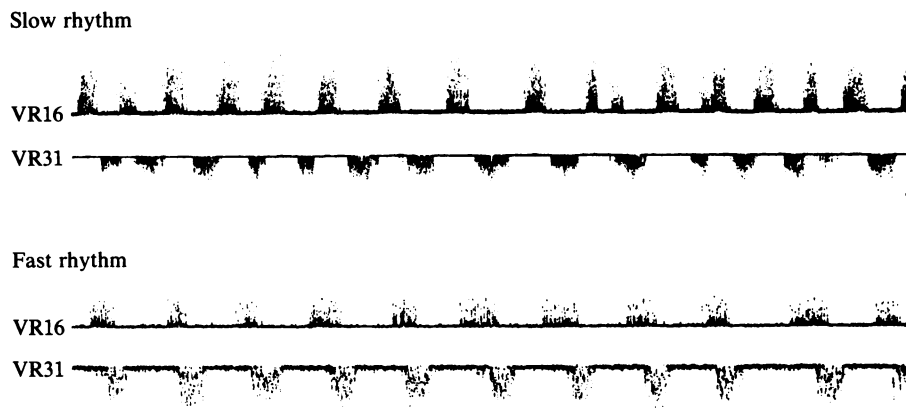

FIG. 2.   Coordinated burst patterns in the right 16th and 31st VRs of a control (not previously transected) spinal cord; first root in the isolated cord piece is designated VR1. Upper traces: the slow rhythm. Lower traces: the fast rhythm, which appeared spontaneously after several minutes. Calibration bars (at right) in all traces represent 1 sec.
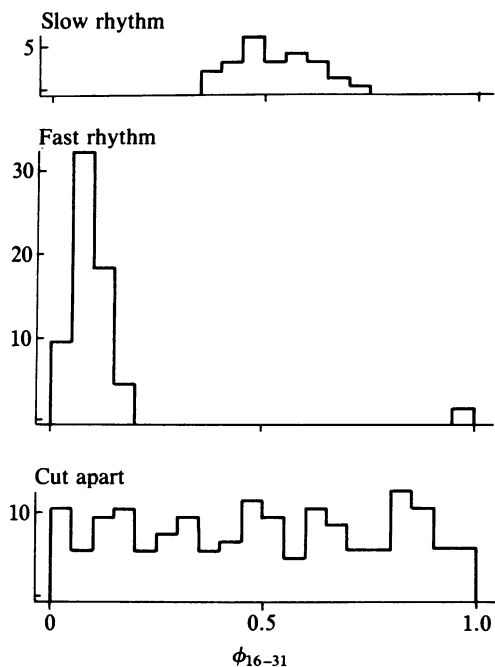
FIG. 3. Distributions of phase lags (ϕ) in recordings from the spinal cord of the control animal illustrated in Fig. 2. Values of 1 and 0 are equivalent and suggest that the two bursts begin simultaneously. Illustrated are the distribution of phase lags for the slow rhythm (*Top*) and the fast rhythm (*Middle*) and the loss of fixed phase lag during fast bursting after the cord was cut between the electrodes (*Bottom*).

DISCUSSION

These experiments show that spinal cord regeneration in the lamprey is accompanied by a return of coordination between the VR discharges from segments above and below the site of
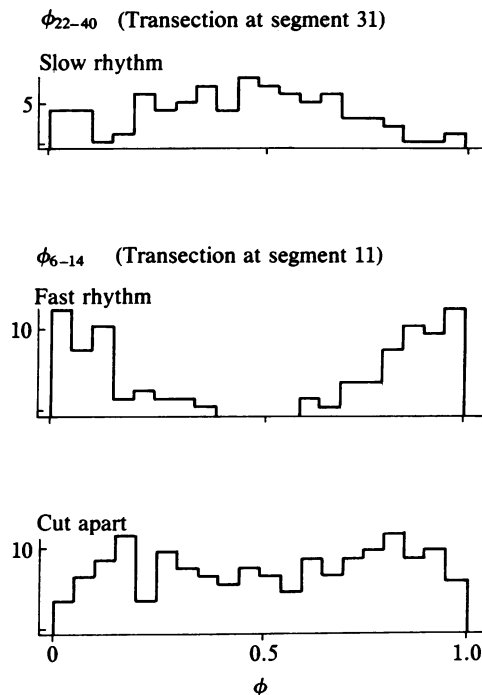
FIG. 5. Nonrandom distributions of phase lags (ϕ) between VRs on opposite sides of the healed injury site in the animal illustrated in Fig. 4. Following retransection at the original scar, the phase lags became randomly distributed (*Bottom*).

injury. Axonal regeneration accompanies behavioral recovery in several nervous system pathways of fish and amphibians. Examples include the retinotectal pathway (13), the auditory system (14), cutaneous sensation following dorsal rhizotomy (15), and free swimming after spinal transection (16–18). However, recovery of a stereotyped behavior such as swimming need not result from true synaptic regeneration.
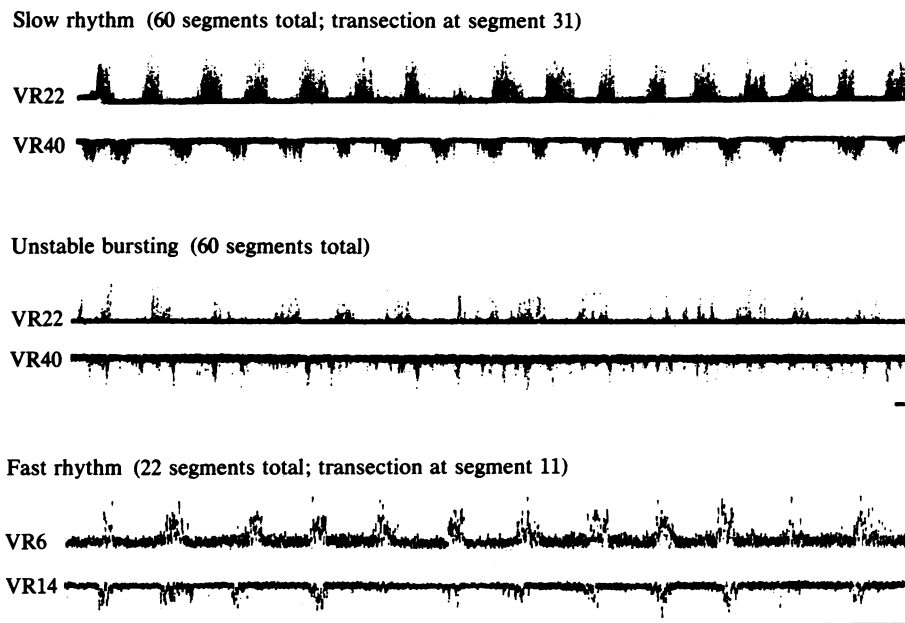
Slow rhythm (60 segments total; transection at segment 31)

VR22

VR40

Unstable bursting (60 segments total)

VR22

VR40

Fast rhythm (22 segments total; transection at segment 11)

VR6

VR14

FIG. 4. VR recordings from an animal that had recovered behaviorally from complete spinal transection. Top traces: the slow rhythm recorded simultaneously from the right 22nd root, which is rostral to the transection scar, and the right 40th root, which is caudal to the scar. The scar is at segment 31. Note the coupling between the two VRs. Middle traces: activity typical of that recorded after the slow rhythm has disappeared. Those cords which had no stable slow rhythm showed a similar pattern. Bottom traces: the preparation has been trimmed to 22 segments, unveiling the fast rhythm. The recording electrodes have been repositioned on the VRs on opposite sides of the healed transection scar. Note that, although not as tightly coupled as in control animals, the bursts from segments by a healed transection scar are still well coordinated. Calibrations bars represent 1 sec.

Coordination between segments across the transection might be mimicked by passive motion of the body below the lesion in response to the oscillation of rostral musculature. Coordination could also be mediated by mechanoreceptors in the spinal cord (19) or by peripheral sensory feedback, even in the absence of axonal regeneration. Alternatively, an intraspinal but nonsynaptic mechanism for motor recovery might involve nonspecific release of transmitters into the extracellular space by the regenerated neurites, with subsequent activation of the intrinsic segmental burst generators. In the present study, isolating the cord eliminated input from mechanoreceptors and other sensory receptors and excluded the possibility of passive mechanical drive. Moreover, nonsynaptic transmitter release would not provide the rapid and precise temporal information necessary for the observed stable phase delays. Therefore, regeneration of functional synapses must have accounted for the recovery of coordinated locomotor activity. It should be stressed that neither the identity nor the specificity of the reconnections is addressed by these experiments. It is possible that, given the highly distributed nature of the lamprey intersegmental coordinating system (unpublished data), regenerated axons may synapse fairly nonspecifically and still be functionally connected within the proper system.

Isolated, previously transected spinal cords did not show the stable fast rhythm unless the number of segments around the scar was reduced to 20–60. The rationale for this procedure follows from a theoretical consideration of the behavior of coupled oscillators (20) as applied to empirical observations on fictive swimming in lampreys. In the lamprey CPG for locomotion, each spinal segment or small group of segments behaves as a neural oscillator (9). The entire CPG can be viewed as a chain of coupled oscillators, each of which has a different endogenous burst frequency which varies as a nonlinear function of location along the spinal cord. The frequency of a group of segments is generally intermediate between the frequencies of the separated rostral and caudal groups. From mathematical modeling (20), it is known that oscillators with very different frequencies require strong coupling to entrain each other. Weak coupling can only entrain oscillators with very similar frequencies. The regenerated coupling is probably not as strong as that in the untransected cord. Therefore, only groups of segments having similar frequencies could be entrained across the lesion site. By reducing the length of the spinal cord piece, the range of frequencies of the individual oscillators is reduced, thus allowing entrainment by a weaker coupling. In intact animals, sensory feedback could increase the coupling and thus explain why normal-appearing swimming is recovered following transection. A more complete explanation appears elsewhere (21).

A common feature of vertebrate locomotor CPGs is that they are composed of a distributed chain of coupled, but relatively autonomous, subunits located at the level of the motorneurons they control (22). Theoretically, return of coordinated locomotion after spinal transection might result from short-distance connections formed between the subunits adjacent to the site of the lesion. Recent developments in peripheral nerve bridge grafting and fetal tissue transplants have demonstrated the intrinsic capacity of mammalian central neurons to regenerate over limited distances within the central nervous system (23, 24). The present findings suggest that such regeneration might suffice to restore some coordinated motor function, since a similarly limited distance of axonal regeneration in the lamprey (3, 6) is adequate to restore coordination of the locomotor CPG subunits across a healed transection.

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