

## RECOVERY OF FUNCTION, PERIPHERAL SENSITIZATION AND SENSORY NEURONE ACTIVATION BY NOVEL PATHWAYS FOLLOWING AXONAL INJURY IN *APLYSIA CALIFORNICA*

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### Summary

Recovery of behavioural and sensory function was examined following unilateral pedal nerve crush in *Aplysia californica*. Nerve crush that transected all axons connecting the tail to the central nervous system (CNS) eliminated the ipsilateral tail-evoked siphon reflex, whose sensory input travels in the crushed tail nerve (p9). The first reliable signs of recovery of this reflex were observed within 1 week, and most animals displayed tail-evoked siphon responses within 2 weeks. Wide-dynamic-range mechanosensory neurones with somata in the ventrocaudal (VC) cluster of the ipsilateral pleural ganglion exhibited a few receptive fields (RFs) on the tail 3 weeks after unilateral pedal nerve crush, indicating that the RFs had either regenerated or been reconnected to the central somata. These RFs were smaller and sensitized compared with corresponding RFs on the contralateral, uncrushed side. Centrally conducted axon responses of VC sensory neurones to electrical stimulation distal to the nerve crush site did not reappear until at least 10 days after the crush. Because the crush site was much closer to the CNS than to

the tail, the failure of axon responses to be restored earlier than the behavioural responses indicates that early stages of reflex recovery are not due to regeneration of VC sensory neurone axons into the tail. Following nerve crush, VC sensory neurones often could be activated by stimulating central connectives or peripheral nerves that do not normally contain the sensory neurone's axons. These results suggest that recovery of behavioural function after nerve injury involves complex mechanisms, including regenerative growth of axotomized VC sensory neurones, sensitization of regenerating RFs and sprouting of VC sensory neurone fibres within the CNS. Furthermore, the rapidity of behavioural recovery indicates that its initial phases are mediated by additional mechanisms, perhaps centripetal regeneration of unidentified sensory neurones having peripheral somata, or transient reconnection of proximal and distal stumps of axotomized VC cells.

Key words: axotomy, regeneration, nociceptor, siphon reflex, hyperexcitability, sprouting, *Aplysia californica*.

### Introduction

Vertebrate and invertebrate animals often react to peripheral injury with adaptive neural reactions that restore function to the damaged area and protect it from further injury (reviewed by Walters, 1994). In principle, various mechanisms could contribute to these adaptive reactions, including regrowth of transected sensory axons into peripheral receptive fields (RFs) (Weddell *et al.* 1941; Diamond *et al.* 1976; Van Essen and Jansen, 1977), reconnection of regenerating neurites to surviving axons in distal nerve stumps (Carbonetto and Muller, 1977; Bittner, 1991), collateral sprouting of neighbouring sensory neurones into tissue denervated by injury (Van Essen and Jansen, 1977; Nixon *et al.* 1984), enhancement of the sensitivity of surviving sensory branches (Reeh *et al.* 1987; Billy and Walters, 1989), facilitation of the synapses of sensory

neurones innervating the injured region (Walters, 1987) and priming of specific motor systems that protect it (e.g. Woody and Black-Cleworth, 1973; Carew and Kandel, 1977; Erickson and Walters, 1988; Frost *et al.* 1988).

How are the various neural reactions to peripheral injury integrated, and what are their respective contributions to adaptive behaviour after injury? These complex questions can be addressed advantageously in relatively simple invertebrate preparations in which the nociceptive and defensive functions of identified neurones and neural networks can be at least partially defined. As a first step, we have begun to characterize potentially adaptive reactions of nociceptive sensory neurones to peripheral nerve injury in the marine snail *Aplysia californica*. A homogeneous, tightly packed cluster of about

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200 sensory neurone somata constitutes the ventrocaudal (VC) cluster in each pleural ganglion. The VC cells are likely to be primary mechanosensory neurones because their responses to tactile stimulation are not abolished by blocking chemical synapses in the periphery or the CNS (Walters *et al.* 1983). Collectively, they innervate the entire ipsilateral surface of the animal, except for the mantle cavity (Billy and Walters, 1987). The VC cells have a wide dynamic range, being activated most intensely by noxious, crushing stimuli, but also responding weakly to innocuous pressure (Walters *et al.* 1983; Clatworthy and Walters, 1993).

Because the VC cells can be reliably identified by appearance alone, and they display considerable plasticity, these sensory neurones have been studied intensively using cellular paradigms that model learning and memory (reviewed by Byrne *et al.* 1993; Walters, 1994; Krasne and Glanzman, 1995). However, the largest, longest-lasting enhancement of excitability and synaptic transmission has been found after injury to either the receptive field of a VC cell or the nerve containing its peripheral axon (Walters, 1987; Billy and Walters, 1989; Walters *et al.* 1991). Because such injury often disconnects sensory neurones from their RFs, we wondered whether these sensory neurones would re-establish their peripheral RFs after axonal transection. Given that axons of molluscan interneurons and motor neurones readily regenerate (e.g. Janse *et al.* 1979; Fredman, 1988; Bulloch and Ridgway, 1989), it seemed likely that transection of the peripheral axons of sensory neurones in *Aplysia californica* would eventually lead to recovery of function *via* regenerative growth of damaged axons. However, the great distance between VC sensory neurone RFs and their centrally located somata (often more than 10 cm) indicated that recovery of sensory function dependent upon transport of materials to regenerating neurites from the soma must be extremely slow. Thus, we wondered whether recovery of sensory function occurs before transected VC sensory neurone axons have time to regenerate, which would indicate that processes other than regrowth of these axons are involved during the early stages of recovery.

Here we address several basic questions that must be answered before specific contributions of different mechanisms to recovery of behavioural function can be determined in this system. First, do behavioural responses eliminated by nerve injury eventually recover? Second, do VC sensory neurones re-establish their peripheral RFs after their axons have been transected by nerve injury? Third, how does the rate of recovery of VC sensory neurone function compare with the time course of behavioural recovery? In particular, do behavioural responses recover before there is time for regrowth of VC sensory neurone axons to the periphery? Fourth, does axonal transection induce potentially adaptive alterations of peripheral processes of VC sensory neurones in addition to any regeneration that occurs? These results, some of which have been reported in abstract form (Dulin and Walters, 1993; Dulin *et al.* 1994), suggest a number of testable hypotheses about specific recovery mechanisms. The accompanying paper

(Steffensen *et al.* 1995) begins to test two of these hypotheses, demonstrating that peripheral regeneration and central sprouting of VC sensory neurone axons accompany later phases of behavioural recovery after nerve injury.

### Materials and methods

*Aplysia californica* Cooper (Gastropoda; Opisthobranchia) (80–300 g) were obtained from Alacrity Marine Biological Services (Redondo Beach, California) and the University of Miami *Aplysia* Research Facility (Miami, Florida). Animals were maintained in artificial sea water (ASW, Instant Ocean) at 17–19 °C and twice per week were fed sufficient romaine lettuce to maintain a relatively constant body mass.

In each experiment, pedal nerves were injured about 1 cm from one of the pedal ganglia (Fig. 1, see also Fig. 7), using previously described methods (Walters *et al.* 1991; Clatworthy and Walters, 1994). Briefly, the animal was anaesthetized by cooling to 1–2 °C and injecting ice-cold isotonic MgCl<sub>2</sub> (equivalent to about 30 % of body mass). In most experiments, all the major pedal nerves except p1 were crushed as they exited one of the pedal ganglia. In some experiments, p9 alone was cut on one side and p1 alone was cut on the other (see Fig. 7). Recovery of behavioural function following unilateral crush of pedal nerves was monitored by examining the tail-evoked siphon reflex (see Fig. 2A; Walters and Erickson, 1986; Hickie and Walters, 1995). To permit an unobstructed view of the siphon, the enveloping parapodia were surgically removed 1–2 cm above their base 1 day before unilateral pedal nerve crush (after anaesthetizing the animals in an ice-cold ASW bath for about 15 min). Siphon responses were tested at 2–3 day intervals. Two types of test stimuli were delivered to the middle of each side of the tail: a moderate intensity pinch with dulled no. 5 forceps (tip diameter 0.4 mm) or progressively stronger trains of shock (50 Hz, 200 ms trains of 10 ms pulses at 0, 20, 40, 80, 160 and 320 mA). The shocks were delivered between a focal (0.6 mm tip diameter) unipolar silver wire electrode lightly touching the skin and a concentric silver wire 2.5 mm behind the tip. These brief stimuli rarely produced ink or opaline release and left no obvious signs of tissue damage. The trains were applied at approximately 10 s intervals until an unambiguous siphon response was observed or until 320 mA had been delivered. To ensure that responses due to possible spread of stimulus current from the nerve-crushed side to the contralateral side of the tail were not included, we excluded from our analysis responses to stimulus intensities greater than 80 mA (see Results).

Most of the behavioural tests were performed by a person unaware both of the side on which the nerve had been crushed and of the purpose of the study. Behavioural measures were restricted to observations of the occurrence or lack of occurrence of an evoked siphon movement in response to each stimulus, the type of siphon response (flare, constriction or withdrawal; see Fig. 2A and Walters and Erickson, 1986; Hickie and Walters, 1995) and a subjective rating of the intensity of the response (weak, moderate or strong). In this

initial study, we were primarily concerned with determining the latency for recovery of reliable siphon responses. To optimize our chances of observing early recovery, our experimental design incorporated two features that could confound interpretations of changes in siphon response intensity: (1) modifications triggered by surgical removal of the parapodia and (2) the frequent delivery of intense test stimuli. Because of these potential artifacts, we did not attempt to measure or analyze changes in the magnitude or duration of siphon responses. We did not test possible effects of prior parapodial surgery on any of the behavioural or physiological phenomena examined.

Electrophysiological tests were performed on animals killed 1–53 days after unilateral nerve crush. Animals were dissected after being anaesthetized with an injection of ice-cold isotonic  $MgCl_2$  equal to 50% of their mass. Each pleural ganglion was desheathed in a 1:1 solution of isotonic  $MgCl_2$  and ASW, washed five times with ASW, and left for 1 h before intracellular recording began. Sensory neurones in each pleural VC cluster were impaled with microelectrodes (10–20 M $\Omega$ ) filled with 3 mol l<sup>-1</sup> potassium acetate and 6 mmol l<sup>-1</sup> Fast Green dye (Sigma). Nerves (p9 and either p1 and p8 or the pleural–abdominal or pleural–cerebral connectives; Fig. 1) were stimulated with 2 ms pulses through suction electrodes

placed 2–3 cm from the VC cluster on the pedal nerves and 3–5 mm from the VC cluster on the central connectives. During nerve stimulation, the ganglion was bathed in a solution containing 0.1 mmol l<sup>-1</sup>  $Ca^{2+}$  (1% of the normal concentration) in order to block chemical synaptic transmission. Stimulus current was approximately four times the amount necessary to evoke antidromic spikes in large, unidentified pleural ganglion neurones adjacent to the VC cluster.

RFs were examined in a semi-intact preparation consisting of the tail attached to separate pairs of pleural–pedal ganglia (Fig. 3A; Walters, 1987). RFs were mapped by tapping the tail with a stiff von Frey hair while recording from VC sensory neurones in the p9 region of the cluster (see Fig. 7B). Once the centre of an RF had been found, its threshold was determined using a series of von Frey hairs of decreasing stiffness. The area was estimated from careful drawings of each field using previously described methods (Billy and Walters, 1989). The number of cells sampled (6–8, with equal numbers on each side in each animal) was limited in order to minimize possible alterations, such as tissue damage and sensitization, produced by the testing procedure. Consequently, the matched samples provided an estimate of the size of the larger fields on each side, rather than the average size of all the fields.

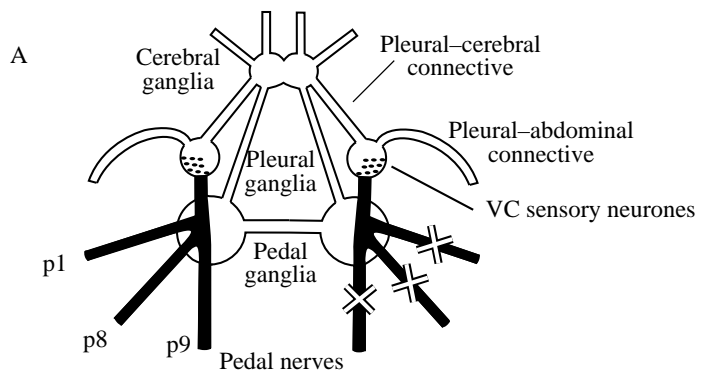
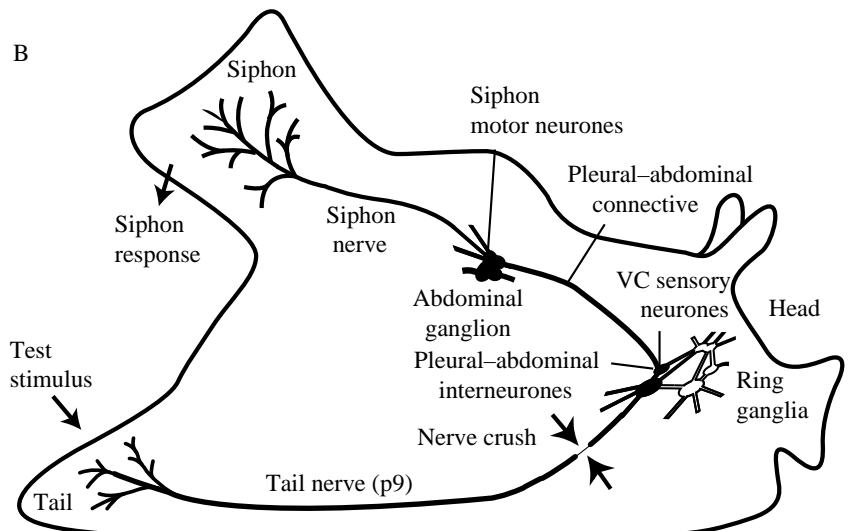


Fig. 1. Pathways mediating the tail-evoked siphon reflex, and the sites of nerve crush. (A) Schematic diagram of ring ganglia (excluding the buccal ganglia) showing ipsilateral pedal nerve crush. Only three of the nine major nerves leaving each pedal ganglion are shown. In most experiments, all the major pedal nerves on one side were crushed (indicated by crosses), except for nerve p1. The sensory neurone somata in the pleural ganglion ventrocaudal (VC) cluster are indicated by dots. The nerves containing axons of the VC sensory neurones are darkened. (B) Neural pathways mediating the tail-evoked siphon reflex. Only the left p9 nerve, left pleural–abdominal connective and siphon nerve are shown. Interneurons relaying excitation from VC tail sensory neurones to LFSB siphon motor neurones have been found in the pleural ganglia (Walters *et al.* 1982; Cleary and Byrne, 1993).



Statistical comparisons between control and nerve-crushed sides were evaluated with paired, two-tailed *t*-tests, using the average values on each side per animal as individual data points. Frequency comparisons were evaluated with a  $\chi^2$ -test.

**Results**

*The tail-evoked siphon reflex begins to recover after 1–3 weeks*

Unilateral pedal nerve crush eliminated centrally mediated defensive responses evoked by tail stimulation on the crushed side. Thus, during the week following nerve crush we were unable to elicit ink or opaline release, escape locomotion or siphon responses by intense pinching of the side of the tail that had been disconnected from the CNS. The tail withdrawal reflex, which is mediated in part by peripheral pathways (Walters, 1987), remained. To investigate recovery of sensory function after VC sensory neurone axons had been transected by pedal nerve crush, we focused on siphon responses.

Even weak stimulation of the tail normally causes a

characteristic reflexive response of the siphon, flaring and bending towards the tail (Fig. 2A; see Walters and Erickson, 1986). As shown in Fig. 1B, the tail-evoked siphon reflex is mediated by well-defined central pathways (Hickie, 1994) in which the sensory axons and motor axons are located in separate nerves (sensory axons in the tail and motor axons in the siphon nerves). Thus, crushing the tail nerve, or other pedal nerves, does not damage the axons of the siphon motor neurones, and recovery of the tail-evoked siphon reflex after p9 crush should depend upon recovery of sensory function rather than motor function. Indeed, immediately after pedal nerve crush and throughout the experiment, siphon responses were consistently evoked from the contralateral side of the tail (Fig. 2B), demonstrating that the motor pathway underlying this reflex was intact.

In contrast, unilateral pedal nerve crush eliminated all tail-evoked siphon responses on the crushed side until 7 days after the nerve crush (Fig. 2B). The only exceptions were a few responses evoked by the most intense shocks (320 mA). We surmised that these responses were probably due to current

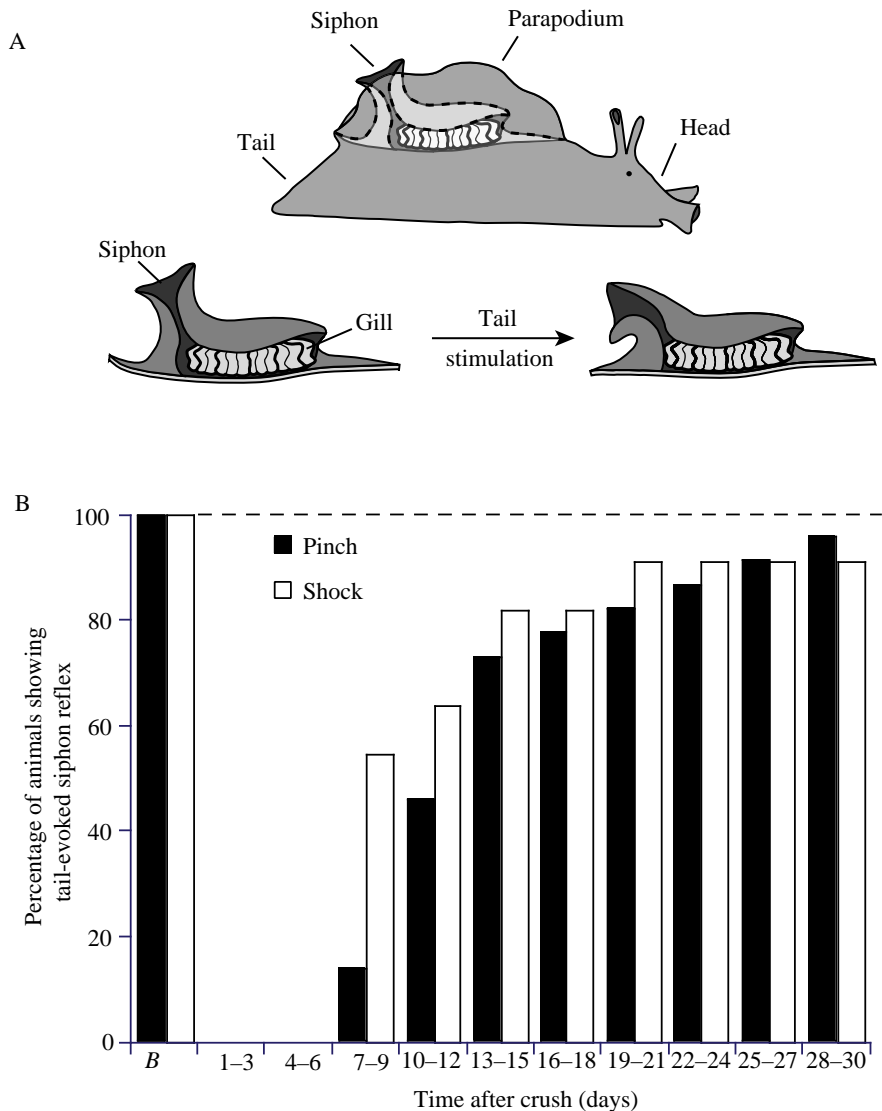


Fig. 2. Recovery of the siphon reflex elicited by cutaneous stimulation of the side of the tail ipsilateral to pedal nerve crush. (A) The tail-evoked siphon reflex. Tail stimulation causes the siphon to bend towards the tail and flare open. Normally the siphon is obscured by the right and left parapodia. Prior to nerve crush or cut, both parapodia were surgically excised 1–2 cm above their insertion into the body wall, providing an unobstructed view of the siphon during the remainder of the experiment. (B) Proportion of animals displaying a tail-evoked siphon reflex before and after unilateral p9 crush. The tail test stimulus was either a light pinch (filled bars, *N*=22 animals) or shock (open bars, *N*=11). Baseline (B) tests were conducted 2 days before nerve crush. The dashed line represents the percentage of animals that responded to stimulation of the control side of the tail on each test.

spread to the control side of the tail. In particular, shocks of 160 mA or greater produced a strong tail contraction which persisted for many minutes, making it difficult to avoid delivering subsequent stimuli near the midline. The observation that no tail-evoked siphon responses were elicited during the first week by pinching the nerve-crushed side of the tail also indicated that the few responses obtained in response to strong shock were due to inadvertent contralateral stimulation. To exclude responses that may have been evoked by current spread to the contralateral side of the tail, we have confined our analysis to shocks of 80 mA or less.

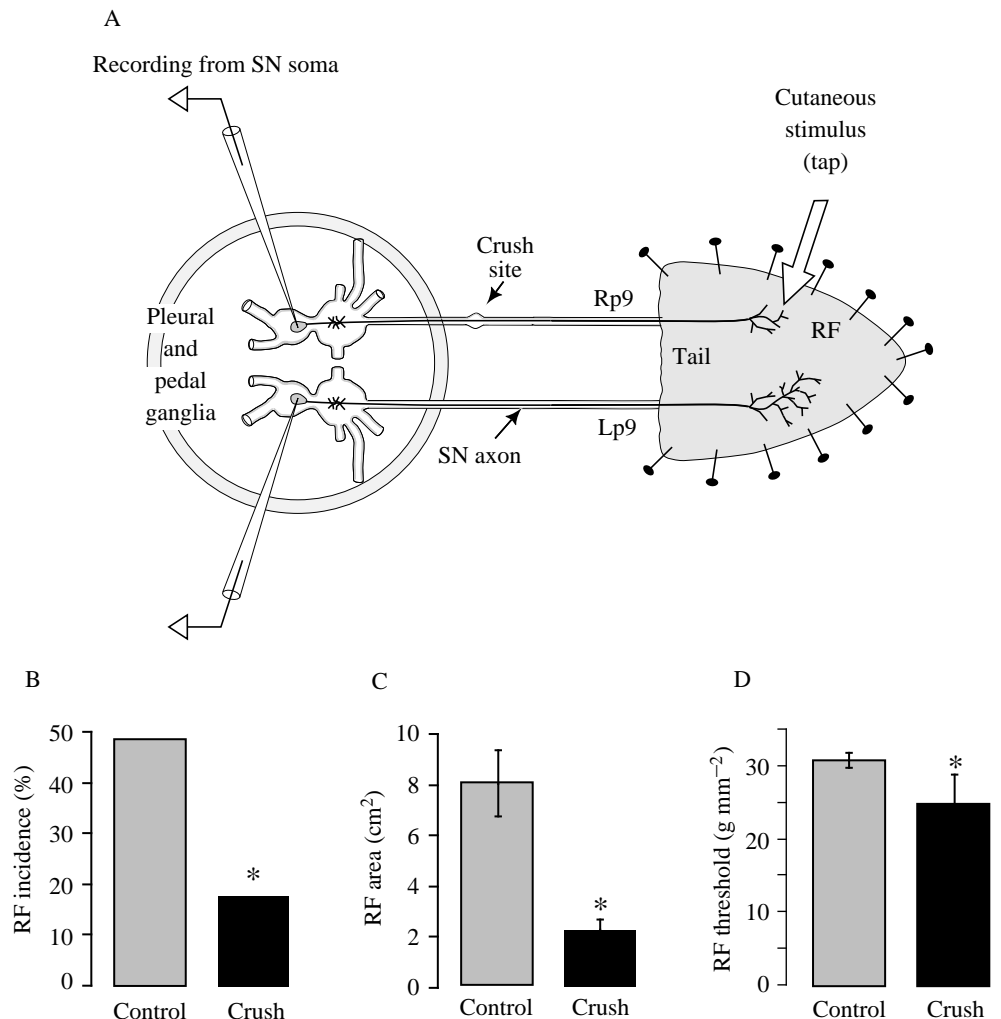
The mean latency for the first reliable siphon response to tail stimulation was  $12.8 \pm 1.2$  days (S.E.M.; pooling the responses to tail pinch,  $N=22$ , and focal tail shock,  $N=11$ ). As can be seen in Fig. 2B, responses to ipsilateral tail shock were initially more likely than responses to ipsilateral tail pinch. The proportion of animals displaying a tail-evoked siphon response to either stimulus then progressively increased between 1 and 3 weeks after unilateral pedal nerve crush. Measurements of the threshold for siphon responses evoked by tail shock showed considerable variation during recovery, even in the same animal, and no significant overall effects of nerve crush on reflex threshold were found. Three of the 33 animals showed

no reflex recovery during the 30 day test period. During the initial stages of recovery, the evoked siphon responses on the nerve-crushed side, although unmistakable, were clearly weaker than those on the control side. Later the responses strengthened, but even 3–4 weeks after nerve crush the tail-evoked siphon responses on the crushed side sometimes appeared weaker than the responses on the uncrushed side. In two of the 33 animals, the earliest siphon responses differed qualitatively from the typical tail-evoked reflex (Fig. 2A), instead resembling the constricting response normally elicited by head stimulation (Walters and Erickson, 1986). In both cases, the constricting response to tail stimulation converted to the normal flaring response after about 4 days.

#### *Sensory neurone RFs show partial restoration and sensitization 3 weeks after nerve crush*

Our nerve crush procedure transected all axons within the crushed nerve (Steffensen *et al.* 1995). To determine whether pleural VC sensory neurones showed functional recovery following axonal transection, we used a semi-intact preparation (Fig. 3A) to test for RFs on the tail 21–25 days after unilateral pedal nerve crush. In this preparation, the tail was connected to each pair of pleural–pedal ganglia solely by the ipsilateral

Fig. 3. Restoration and sensitization of VC sensory neurone receptive fields (RFs). (A) Diagram of the semi-intact preparation used to investigate the responses of pleural VC sensory neurone somata to mechanical stimulation of the tail. The soma in the pleural ganglion, presynaptic branches in the pedal ganglion, axon in p9 and peripheral arborization in the tail are indicated schematically for a single sensory neurone on each side. In this example, the right pedal nerve was crushed, as indicated by the typical swelling at the crush site. SN, sensory neurone. (B) Presence of VC cell RFs on the nerve-crushed side 3 weeks after crush. The percentage of sensory neurones tested in the tail region of the VC cluster that had an RF on the tail was lower on the crushed than on the control side ( $P < 0.001$ ,  $N=30$  and 29 cells, respectively). (C) Mean size of the largest RFs on the crushed and uncrushed sides ( $P < 0.001$ ,  $N=4$  animals). On both sides, the rapid sampling procedure was selective for larger fields (see Materials and methods). (D) Mean von Frey hair thresholds on the crushed and uncrushed sides ( $P < 0.05$ ,  $N=4$ ). Error bars represent the S.E.M. Asterisks indicate values significantly different from the control values.



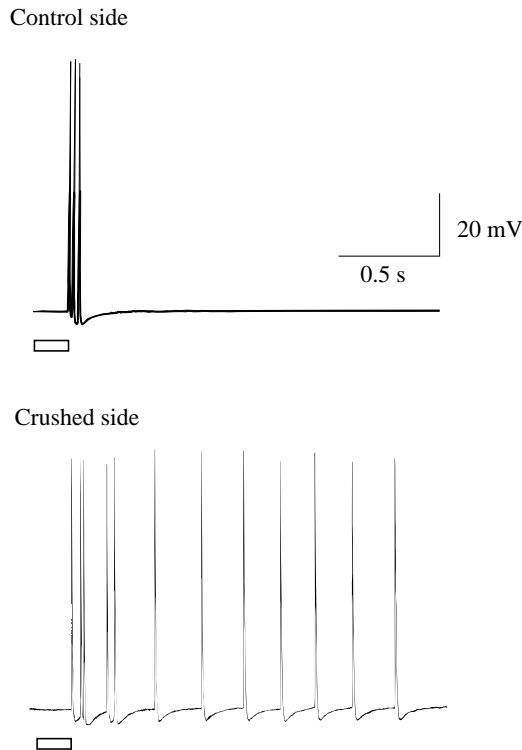


Fig. 4. Afterdischarge in response to brief tail stimulation 21 days after pedal nerve crush. (A) Response of sensory neurone on the uncrushed side to a tap (open bar, approximately 200 ms) delivered with a  $70 \text{ g mm}^{-2}$  von Frey hair to the ipsilateral surface of the tail. (B) Response of a sensory neurone on the crushed side to the same stimulus. Note the afterdischarge, which lasts about 1.5 s.

tail nerve (p9). Systematic probing of the tail with a stiff von Frey hair (exerting a pressure of approximately  $70 \text{ g mm}^{-2}$ ) elicited action potentials in one or more VC sensory neurones in three of four preparations examined on the previously crushed side, and in all four preparations on the uncrushed side. In each VC cluster, 6–8 somata were surveyed (out of 20–30 in the tail/p9 region; Walters *et al.* 1983), with the same number being sampled on each side in each animal. As shown in Fig. 3B, significantly fewer RFs were found on the crushed side (five of 30 cells tested) than on the uncrushed side (14 of 29 cells;  $P < 0.001$ ). This difference suggests that approximately one-third of the sensory neurones had re-established RFs on the nerve-crushed side. The fields sampled on the crushed side were significantly smaller than those on the control side (Fig. 3C,  $P < 0.001$ ), so some fields may have been missed on the crushed side. No fields on the uncrushed or crushed side were found to cross over to the contralateral side of the tail. After the stiff von Frey hair had been used to locate and map the RF of a VC sensory neurone, progressively softer von Frey hairs were used to determine its mechanical threshold. Interestingly, the mechanosensory thresholds of the RFs on the previously crushed side were significantly lower than those on the uncrushed side (Fig. 3D,  $P < 0.05$ ). However, one of the eight RFs on the crushed side had an unusually high threshold,

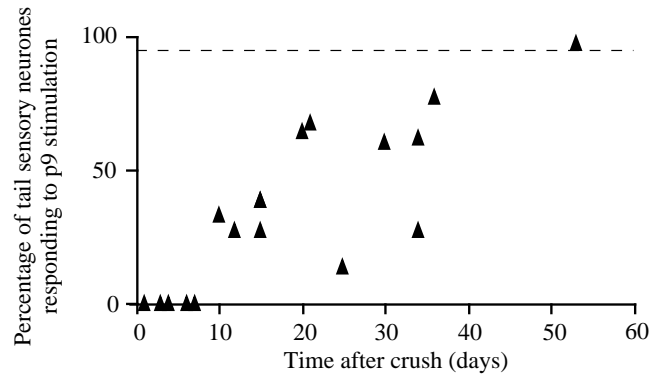


Fig. 5. Recovery of tail sensory neurone responses to stimulation of nerve p9 distal to the crush site. Each triangle represents data from a single animal; specifically, the mean percentage of sensory neurones sampled in the p9 region of the VC cluster (see Fig. 7B) that displayed centrally conducted action potentials evoked by p9 stimulation on the side that had undergone unilateral pedal nerve crush. The dashed line represents the average percentage of cells in the p9 region that responded on the control side. 4–10 neurones were sampled per cluster in each animal (total cells sampled in all animals, 102 on each side).

requiring a von Frey stimulus of  $60 \text{ g mm}^{-2}$ . Of the seven fields with low thresholds, three displayed an afterdischarge that continued for up to 1 s after the end of the stimulus (Fig. 4). None of the RFs on the uncrushed side showed an afterdischarge to any of the test stimuli.

In the study just described, we did not test excitability of the soma. However, in separate experiments performed during the same period, 10 of 38 preparations showed an afterdischarge following brief (20 ms) suprathreshold depolarization of the soma. As with that elicited by tail stimulation, the afterdischarge elicited by soma stimulation was much more likely to occur in sensory neurones with previously crushed axons than in contralateral control cells (cf. Walters *et al.* 1991). In nine animals in which an afterdischarge elicited by soma stimulation was observed, nine of 28 cells tested on the crushed side showed an afterdischarge whereas only one of 28 cells on the uncrushed side showed an afterdischarge ( $P < 0.01$ ).

#### *Sensory neurone responses to tail nerve stimulation recover slowly after nerve crush*

Whereas behavioural responses to tail stimulation began to recover 1–2 weeks after nerve injury (Fig. 2B), VC sensory neurone RFs were difficult to find between 3 and 4 weeks after nerve injury (Fig. 3B). This suggested that there may be a discrepancy between the rate of behavioural recovery and the rate of recovery of VC sensory function. To see whether behavioural responses recover before there is time for regrowth of VC sensory neurone axons to the periphery, we examined the time course of recovery of electrophysiological responsiveness of VC cells to electrical stimulation of the ipsilateral tail nerve (p9) delivered 1–2 cm distal to the crush site. These distinctive sensory neurones can be identified unambiguously by visual and

electrophysiological properties in the absence of RF stimulation (Walters *et al.* 1983). Under normal conditions, shocking p9 evokes centrally conducted action potentials in 85–95% of sensory neurones in the 'tail/p9 region' of the somatotopically organized pleural VC cluster, with the remainder of the VC cells being activated by shock to other pedal nerves (Walters *et al.* 1983; Billy and Walters, 1987; see Fig. 7B). Because our nerve crush procedure transected all axons within the crushed nerve (Steffensen *et al.* 1995), we predicted that the crush would eliminate all action potentials in the sensory neurone somata evoked by distal nerve stimulation.

Following unilateral crush of pedal nerves (including p9), we were initially unable to evoke centrally conducted axon responses in any pleural sensory neurone somata by intense ipsilateral p9 shock (Fig. 5). In contrast, 95% of the sensory neurone somata sampled in the tail/p9 region of the VC cluster on the uncrushed side of the animal were activated by shocking the corresponding p9 nerve. On the crushed side, responses to ipsilateral p9 shock were first observed 10 days after nerve crush (Fig. 5). It should be noted that this latency was the same or longer than that for behavioural recovery, even though the nerve stimulus was only 2–3 cm from the CNS, whereas behavioural recovery required reconnection to the periphery more than 10 cm from the CNS. The proportion of sensory neurones sampled in the tail/p9 region that was activated by p9 shock tended to increase slowly on later tests. Linear regression on the points in Fig. 5 indicated that the proportion of sensory neurones in this region activated by p9 shock returns to the control level about 50 days after crush (slope = 1.93% day<sup>-1</sup>,  $r=0.89$ ).

#### *Sensory neurones can be activated via novel pathways after axonal injury*

Behavioural recovery after nerve injury is commonly attributed to axonal regeneration (e.g. Griffin and Hoffman, 1993). However, additional mechanisms might contribute, including sprouting of axons into novel nerves and the formation of electrical synapses with novel targets (see Discussion). To see whether mechanisms involving novel sensory pathways could contribute to behavioural recovery after pedal nerve crush, we looked for changes in patterns of electrophysiological responsiveness to electrical stimulation of central connectives and peripheral nerves. In the absence of prior injury, each pleural sensory neurone innervating the tail can only be activated by appropriate stimulation of its RF on the tail or by artificially depolarizing the cell soma or axon, which projects through the ipsilateral tail nerve (p9). The cells do not receive fast excitatory postsynaptic potentials (EPSPs) and are not activated by the slow EPSPs they receive (Walters *et al.* 1983). Thus, intense nerve shock only evokes action potentials when delivered to nerve p9. Our first evidence for injury-induced changes in activating pathways was obtained during the study described above, in which we examined the recovery of tail sensory neurone responses to p9 stimulation. In many of these experiments we also stimulated two central pathways which, under normal conditions, lack axons of tail

sensory neurones: the ipsilateral pleural–abdominal and pleural–cerebral connectives (Walters *et al.* 1983; Billy and Walters, 1987). Fig. 6 shows that stimulation of neither connective activated sensory neurone somata in the tail/p9 region of the VC cluster during the first week after ipsilateral pedal nerve crush. However, between 10 and 54 days after crush, every preparation tested had one or more sensory neurones in this region activated by stimulating either or both connectives (as well as by stimulating p9). The responses to connective stimulation were unlikely to be synaptically mediated because the tests were conducted in a solution containing only 0.1 mmol l<sup>-1</sup> Ca<sup>2+</sup>, which effectively blocks chemical synaptic transmission in *Aplysia californica*. Unlike the responses to p9 stimulation, the responses to connective stimulation did not become progressively more likely over time. Indeed, some of the largest proportions of tail sensory neurones activated by stimulation of central connectives were observed at the earliest points at which the responses appeared.

To see whether expansion of activating pathways would also occur after much more restricted nerve injury, we examined the effects of transecting a single pedal nerve on each side of the animal on the number of sensory neurones that could be

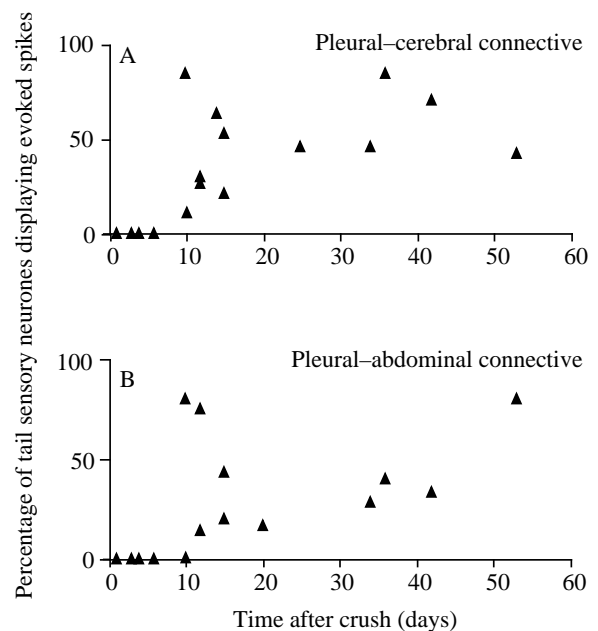


Fig. 6. Activation of sensory neurones by stimulation of normally uninervated connectives following nerve crush. Each triangle represents data from a single animal; specifically, the mean percentage of sensory neurones sampled in the tail/p9 region of the VC cluster that were activated by connective stimulation on the side that had undergone pedal nerve crush. (A) Percentage of sampled cells in each animal with centrally conducted action potentials evoked by pleural–cerebral connective stimulation (total cells sampled in all animals, 96 on each side). (B) Percentage of cells with action potentials evoked by pleural–abdominal connective stimulation (total cells sampled, 90 on each side). No cells in the sampled region were activated by stimulating either connective on the uncrushed side in any animal tested.

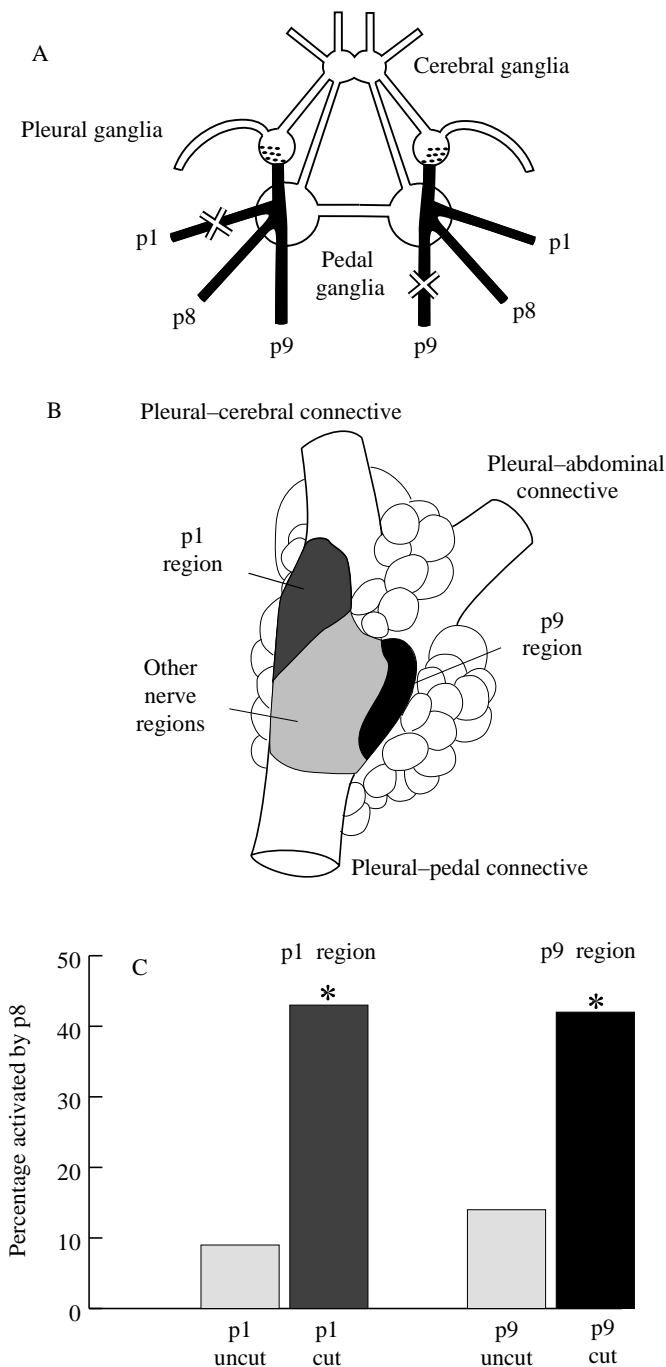


Fig. 7. Increased incidence of centrally conducted action potentials evoked in sensory neurones by stimulation of nerve p8 after cutting nerve p1 or nerve p9. (A) Schematic diagram of p1/p9 nerve cut experiment. In each animal, nerve p1 was cut on one side and p9 on the other. The other nerves were not damaged. During testing, suction electrodes were used to stimulate nerves p1, p8 and p9 on each side. (B) Regions in the pleural VC cluster with somata having their axons in nerve p1 or nerve p9. Somata with axons in all the other nerves (including p8) are primarily found in the remainder of the cluster. (C) Percentage of sampled somata in the p1 and p9 regions of the VC cluster activated by stimulation of nerve p8. The 'uncut' and 'cut' labels refer to the nerve indicated in the label, which was on the same side that was sampled. Asterisks indicate significant differences ( $P < 0.01$ ) from the uncut values.

activated by stimulation of a pedal nerve adjacent to the transected nerve. In each animal, nerve p1 (which innervates the anterior foot) was cut on one side and nerve p9 (which innervates the posterior foot and tail) was cut on the opposite side (Fig. 7A). 3–4 weeks later we stimulated nerve p8 (which innervates the remaining part of the foot; see Jahan-Parwar and Fredman, 1978) as well as p1 and p9 on each side of the body, in solutions containing  $0.1 \text{ mmol l}^{-1} \text{ Ca}^{2+}$  to block chemical synaptic transmission. We looked for evoked action potentials in sensory neurone somata in two distinct regions of each VC cluster. These clusters are somatotopically organized (Walters *et al.* 1983; Billy and Walters, 1987; Zhang *et al.* 1993; Dulin *et al.* 1994), with no overlap in location of the somata of sensory neurones projecting into nerve p1 with those projecting into p9 (Fig. 7B). Furthermore, somata in each of these regions show relatively little overlap with somata of sensory neurones projecting into p8. We found that stimulation of nerve p8 activated only 9% of the 92 somata sampled in the p1 region of the VC cluster on the side with p1 uncut (Fig. 7C). By contrast, the same test stimulus activated significantly more (43%) of the 44 somata sampled in the p1 region when p1 was cut on the same side ( $P < 0.001$ ). Similarly, p8 stimulation activated only 14% of the 59 somata sampled in the p9 region on the side with p9 uncut, but 42% of the 52 somata sampled in the p9 region when the ipsilateral p9 nerve was cut (Fig. 7C;  $P < 0.01$ ). In each case, most of the cells activated by p8 stimulation were also activated by either p1 or p9 stimulation. These results show that an expansion of activating pathways occurs after relatively restricted nerve injury and that this expansion includes adjacent peripheral nerves.

Preliminary observations suggest that nerve injury can also change the RFs for siphon reflexes. In four of the animals given p1 and p9 cuts on opposite sides of the body, we examined the RFs for the characteristic constricting siphon responses produced by p1 (or head) stimulation and the flaring siphon responses produced by p9 (or tail) stimulation (see Fig. 2A and Walters and Erickson, 1986; Hickie and Walters, 1995). For experimental convenience we focused on the dorsal midbody area, especially the base of the parapodia (innervated by p5, p6 and p7), rather than the inaccessible bottom of the foot which is innervated by p8. Like the p8 region, the p5, p6 and p7 regions of the VC cluster show little or no overlap with the p1 and p9 regions of the cluster. Normally, stimulation of the anterior base of the parapodium never elicits siphon flaring, and stimulation of the posterior base of the parapodium never elicits siphon constriction (Hickie, 1994). However, in two of four animals, stimulation of the base of the anterior parapodium on the side with p9 cut sometimes elicited flaring. In three of four animals, stimulation of the base of the posterior parapodium on the side with p1 sometimes elicited siphon constriction. The same responses did not occur on the corresponding locations on the opposite side of the body. These initial observations are consistent with the possibility that nerve cut caused some sensory neurones with axons in the cut nerve to acquire the ability to be activated by stimulation of body regions served by other nerves.



## Discussion

### *Recovery of tail-evoked siphon reflex after tail nerve crush*

Although neural regeneration has been investigated extensively in various vertebrate and invertebrate preparations, cellular mechanisms underlying behavioural recovery after nerve injury are not yet well defined. In gastropod molluscs, where behavioural recovery after brain or nerve lesions has been described (e.g. Moffett and Snyder, 1985; Fredman, 1988; Scott and Kirk, 1992), most cellular analyses have focused on restoration of *motor* pathways (e.g. Murphy and Kater, 1980; Kruk and Bulloch, 1992; Ross *et al.* 1994). Recovery of *sensory* function after nerve injury has not been examined directly in molluscs. In invertebrates, cellular studies of sensory recovery have largely been restricted to leech mechanosensory neurones (Van Essen and Jansen, 1977; Bannatyne *et al.* 1989). The return of tail-evoked siphon behaviour observed in the present study is almost certainly due to recovery of sensory function because the tail-evoked siphon reflex is mediated exclusively by central pathways that receive sensory input through the p9 nerves and send motor output through the siphon nerve (Fig. 1B, Hickie, 1994). The lack of obvious effects of ipsilateral p9 crush on siphon responses evoked by stimulating the contralateral (undamaged) side of the tail confirmed that motor function was not impaired. Nevertheless, we occasionally observed siphon constriction instead of flare being elicited by tail stimulation after nerve crush. These transient, aberrant responses might be due to either motor or sensory alterations. Because of the complete separation of sensory and motor pathways, and the accessibility of identified sensory, motor and interneurons important for the tail-evoked siphon reflex (Walters *et al.* 1983; Frost *et al.* 1988; Cleary and Byrne, 1993; Hickie and Walters, 1995), this system offers considerable promise for defining mechanisms that contribute to behavioural recovery after selective injury to sensory or motor pathways. Indeed, this system was recently used by Noel *et al.* (1995) to examine molecular changes during similar recovery of tail-evoked siphon responses after combined unilateral crush of the pleural–pedal connective and pedal nerves. Our observations of incomplete recovery of this reflex 4 weeks after nerve crush agree with their measurements of depressed siphon response duration 3 and 6 weeks after a crush.

### *Recovery of VC sensory neurone function*

VC tail sensory neurones are known to excite siphon motor neurones mediating the tail-evoked siphon reflex (E. T. Walters, unpublished observations). Therefore, the most obvious hypothesis to explain recovery of the tail-evoked siphon reflex after p9 crush is regeneration of the damaged axons of VC sensory neurones into the tail (Fig. 8). Our sampling 3 weeks after p9 crush indicated that one-third or more of the injured VC tail sensory neurones had peripheral RFs on the tail. The relatively small number and size of the RFs on the recovering side at this time could reflect either (1) that the tested VC cells had regenerating fibres which had not yet completed regrowth of RFs, or (2) that the VC cells had

degenerating distal fibres, perhaps connected to central stumps by gap junctions (see below) that permit passage of action potentials but not large molecules or organelles necessary for long-term maintenance of distal neurites. The first possibility is supported by morphological evidence for regeneration of VC sensory neurone axons (Steffensen *et al.* 1995) and by the slow return of central responses of VC cells to electrical stimulation of p9 distal to the transection site (Fig. 5). The alternative hypothesis (slow degeneration of VC RFs after early formation of gap junctions between central and peripheral stumps of transected axons) predicts that central responses of VC cells to electrical stimulation of p9 distal to the transection site should reappear relatively suddenly and then either remain elevated in frequency of occurrence or slowly decline with time. The slow, progressive increase in frequency of central responses to peripheral stimulation seen over 1–2 months (Fig. 5) suggests that the small number and size of VC cell RFs found 3 weeks after ipsilateral nerve crush reflect incomplete regrowth of regenerating fibres.

### *Multiple mechanisms are likely to underlie reflex recovery*

Our morphological evidence (Steffensen *et al.* 1995) demonstrates that VC sensory neurones, like molluscan motor and interneurons (e.g. Murphy and Kater, 1980; Benjamin and Allison, 1985; Murphy *et al.* 1985; Ross *et al.* 1994), exhibit axonal regeneration after nerve crush. However, two arguments indicate that regenerative growth of VC cell axons from the site of p9 crush into the tail is not solely responsible for the observed recovery of the siphon withdrawal reflex. First, the reflex reappeared as early as (or even earlier than) centrally conducted action potentials in VC sensory neurones evoked by nerve stimulation 2–3 cm distal to the crush site. If sensory input for the tail-evoked siphon reflex were carried exclusively by VC cell axons that had regrown all the way to the tail (6–12 cm from the crush site), the VC responses to nerve stimulation near the crush site should have reappeared much earlier, since the crush site was only 2–3 cm from the VC somata. Second, regrowth beginning at the crush site and continuing to the original RFs would have to proceed at a rate of about  $1 \text{ cm day}^{-1}$  to get to the tail within 10 days (when about half the animals showed reflex recovery). The growth rate would have to be even faster to explain the apparent recovery of tail-evoked siphon responses observed by Noel *et al.* (1995) 3 days after combined connective and nerve crush. These rates greatly exceed previously reported rates of nerve regeneration in molluscs, which range from  $0.4 \text{ mm day}^{-1}$  to  $1 \text{ mm day}^{-1}$  (Murphy and Kater, 1978; Allison and Benjamin, 1985; Kruk and Bulloch, 1992). Moreover, they exceed the rate of transport of cytoskeletal molecules necessary for neurite extension. This slow transport rate ranges between 1 and  $3 \text{ mm day}^{-1}$  at  $37^\circ\text{C}$  in mammals (Griffin and Hoffman, 1993) and is presumably even slower at  $15\text{--}20^\circ\text{C}$  in *A. californica*. An axonal regeneration rate of less than  $1 \text{ mm day}^{-1}$  in *A. californica* was observed by Ross *et al.* (1994), who showed that buccal motor neurones in *A. californica* did not reconnect

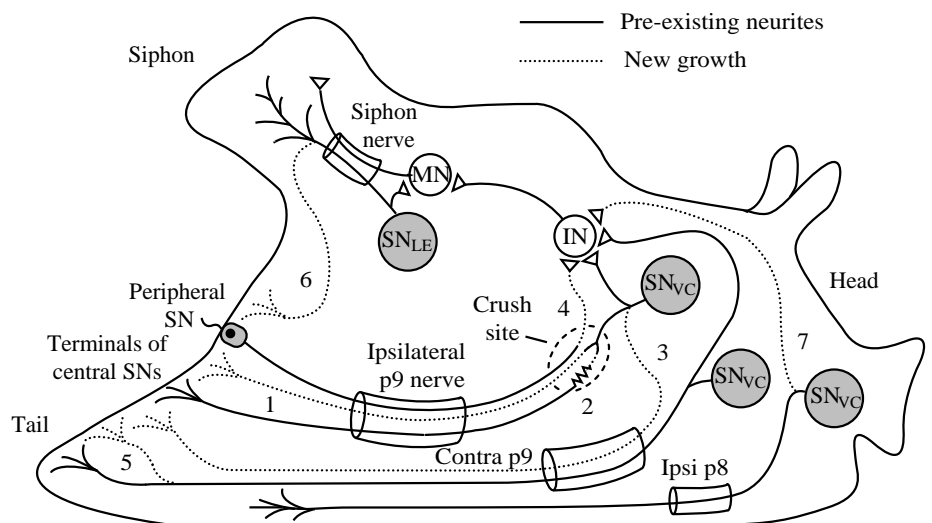
to buccal muscle about 1 cm away until 3–4 weeks after buccal nerve crush.

These considerations, combined with other observations in this initial study, suggest that multiple mechanisms contribute to recovery of the tail-evoked siphon response after unilateral pedal nerve injury. As a first step towards defining these mechanisms, we list seven potential mechanisms that need to be examined further in this system (Fig. 8). Mechanism 1 is regenerative growth of VC cell axons from the site of injury all the way to the tail *via* the crushed p9 nerve. Thus far, this mechanism has received the most extensive support (Steffensen *et al.* 1995). We know that regeneration of VC axons occurs, but we do not yet know its time course or its relative contribution to behavioural recovery at different times after nerve crush. Mechanism 2 is reconnection of regenerating neurites to surviving axons in the distal stump of p9 (see Bittner, 1991). *A. californica* axons, like those in many animals, survive for long periods distal to a point of transection (Ross *et al.* 1994). Although there has been no direct evidence for fusion of proximal and distal stumps in molluscs (as has been occasionally observed in the leech; DeRiemer *et al.* 1983), some molluscan neurones (including *A. californica* motor neurones) are reported to form novel electrical connections after axon injury (Bulloch and Kater, 1982; Cohan *et al.* 1987; Ross *et al.* 1994). This potentially rapid recovery mechanism is discussed further in the accompanying paper. Mechanism 3 is growth of new axons from injured VC cells into aberrant, but undamaged, nerves (e.g. the contralateral p9 or ipsilateral p8 nerve if it is not crushed), which could permit new axons to approach the original RF from a neighbouring area of body wall. This possibility is supported by observations that, after axon crush, VC cell spikes are elicited by stimulation of aberrant connectives and nerves (Figs 6, 7) and by

observations of new neurites growing into aberrant connectives (Steffensen *et al.* 1995).

The remaining mechanisms do not directly involve the injured VC sensory neurones. Mechanism 4 is regeneration of the central axons of sensory neurones possessing peripheral somata (or the central axons of peripheral interneurons that are excited by peripheral sensory neurones). Primary and possibly secondary peripheral sensory neurones are probably numerous in *A. californica* (Xin *et al.* 1992), but any contributions they may make to the tail-evoked siphon reflex are not yet known. This mechanism is of particular interest for the present study because the distance for regenerative growth of peripheral sensory neurones from the site of axon injury to their central targets (approximately 1 cm) was much shorter than the distance for regeneration of VC axons from the injury site to the tail (approximately 10 cm). Consequently, this mechanism could have contributed to early recovery of the tail-evoked siphon reflex. Mechanism 5 is collateral sprouting from VC sensory neurones with adjoining RFs (i.e. VC cells with axons in the contralateral p9 or ipsilateral p8 nerves). Consistent with previous observations of a very strong barrier between RFs on opposite sides of the body (Billy and Walters, 1989), we saw no evidence for sprouting from contralateral VC sensory neurones after ipsilateral p9 crush. However, collateral sprouting from contralateral sensory neurones with peripheral somata, or from *ipsilateral* VC cells, remains an interesting possibility. Mechanism 6, collateral sprouting from centrally located sensory neurones in the abdominal ganglion (LE, rLE, RE or RF cells; Byrne *et al.* 1974; Dubuc and Castellucci, 1991) also remains a possibility. Mechanism 7 is growth of new synapses to interneurons mediating the tail-evoked siphon reflex from undamaged VC sensory neurones with RFs close to the tail. Although we have no direct evidence for this possibility, it

Fig. 8. Potential mechanisms for recovery of the tail-evoked siphon response following unilateral crush of tail nerve (p9). (1) Regeneration of the original axon and arborization by the injured VC sensory neurone. (2) Reconnection of the proximal and distal stumps of the transected VC sensory neurone axon. The resistor symbol represents one potential method of reconnection: the formation of gap junctions between outgrowing sprouts and the distal stump. (3) Growth of a new axon from the injured VC sensory neurone into the undamaged nerve with a contiguous peripheral field (e.g. contralateral p9) and then into the original RF. (4) Regeneration of the injured axon of the sensory neurone with a peripheral soma. (5) Collateral sprouting from the axon of a nearby, undamaged VC sensory neurone, e.g. a contralateral VC tail sensory neurone. (6) Collateral sprouting from a centrally located sensory neurone in the abdominal ganglion which normally innervates the mantle organs (an LE siphon sensory neurone is indicated). (7) Growth of new synapses from a VC sensory neurone with a neighbouring RF (e.g. located in the posterior field of p8) to interneurone-exciting motor neurones that produce tail-evoked siphon response. SN, sensory neurone; MN, motor neurone; IN, interneurone; Contra, contralateral to nerve crush; Ipsi, ipsilateral to nerve crush.



would be consistent with suggestions of extensive neuritic outgrowth in the molluscan CNS after nerve injury (Bulloch and Ridgway, 1989; Steffensen *et al.* 1995) and could be particularly important for peripheral injury in which some VC sensory neurone axons innervating the injured region escape transection.

#### Enhancement of VC cell responsiveness after axonal injury

Previously it was found that unilateral crush of pedal nerves in anaesthetized *A. californica* causes very long-lasting soma hyperexcitability and synaptic facilitation in injured VC sensory neurones (Walters *et al.* 1991; Clatworthy and Walters, 1994). The present results show that regenerating RFs of these sensory neurones, as well as their somata, show increased excitability (Figs 3D, 4), even in the absence of damage to the body wall. Long-lasting RF hyperexcitability also occurs after direct injury to the tail body wall (Billy and Walters, 1989). These observations support the proposal (Walters, 1991; Clatworthy and Walters, 1994) that axon damage and other concomitants of severe peripheral injury trigger a dramatic increase in signalling effectiveness of injured sensory neurones, so that the cells become more sensitive to their peripheral inputs and increase their synaptic output (both by synaptic facilitation and by generation of an afterdischarge in the soma). This cell-wide increase in signalling effectiveness enhances afferent signals from axotomized sensory neurones while their RFs are being restored and from sensory neurones whose RFs were only partially damaged.

An unexpected finding was that, following axonal injury, VC sensory neurones often became responsive to electrical stimulation of central connectives (Fig. 6) or peripheral nerves (Fig. 7C) that do not normally contain axons of the the sensory neurones. The aberrant responses were unlikely to involve chemical synaptic transmission because of the low concentration of  $Ca^{2+}$  in the recording solution. They may reflect, in part, the formation of novel electrical synapses with neurones having axons in different nerves. However, our evidence for injury-induced growth of sensory neurone axons into novel connectives (Steffensen *et al.* 1995) strongly suggests that at least part of the expansion of activating pathways results from outgrowth of new axons from damaged sensory neurones. If so, this growth may represent a mechanism that compensates for severe sensory deficits in an injured region. New axons in different peripheral nerves may provide alternative routes to the vicinity of the original RF, while new growth in the CNS may provide opportunities to form synapses with neurones that can aid in protecting and restoring function to the injured region. These highly accessible sensory neurones with known behavioural functions should permit investigation of fundamental mechanisms underlying behavioural recovery after nerve injury and the relationship of these mechanisms to other forms of plasticity such as learning and memory (Walters *et al.* 1991; Walters and Ambron, 1995).

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