COMPARATIVE ANALYSIS OF HYPEREXCITABILITY AND SYNAPTIC FACILITATION INDUCED BY NERVE INJURY IN TWO POPULATIONS OF MECHANOSENSORY NEURONES OF Aplysia californica

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Accepted 2 February 1994

Summary

Long-term effects of nerve injury on electrophysiological properties were compared in two populations of mechanosensory neurones in Aplysia californica: the J and K clusters in the cerebral ganglia and the VC clusters in the pleural ganglia. Following crush of cerebral nerves containing their axons, the cerebral J/K sensory neurones showed long-term changes that were quite similar to alterations previously described in the VC sensory neurones after either axonal injury or aversive learning. These changes include synaptic facilitation, an increase in soma excitability and spike duration, and a decrease in spike threshold and afterhyperpolarization. In addition, simultaneous crush of both the cerebral and pedal nerves in the same animals produced alterations in the cerebral J/K sensory neurones and pleural VC sensory neurones that were virtually identical. The incidence of hyperexcitability was the same in cerebral J/K and pleural VC sensory neurones when all their axons were crushed, even though the former population includes many neurones that probably have appetitive functions while the latter population appears to be made up exclusively of neurones with defensive functions. Long-term plasticity in both sensory populations failed to occur when nerves lacking axons of the tested neurones were crushed, even when the crush site was very close to the somata of the sensory neurones. This axonal specificity argues against a role for delayed activation of facilitatory interneurones in triggering the plasticity. Several observations are consistent with a triggering role for either (1) intracellular signals released directly by axonal injury or (2) extracellular signals released locally by other axons or injured support cells, or by immunocytes attracted to the injured site.

Introduction

The opisthobranch mollusc Aplysia californica possesses several populations of mechanosensory neurones whose somata are located in discrete, easily recognized clusters within central ganglia. These conveniently situated neurones have provided detailed views of cellular processes involved in the control and modification of behaviour.

Key words: sensitization, axotomy, excitability, synaptic facilitation, Aplysia californica.
The sensory populations that have been examined most intensively are the LE neurones in
the abdominal ganglion, which innervate the siphon and mantle (Byrne et al. 1974), and
the VC neurones in each pleural ganglion, which innervate virtually all the ipsilateral
body surface except for the mantle organs (Walters et al. 1983a, E. T. Walters,
unpublished observations). Both of these sensory populations have been shown to trigger
defensive reflexes (Byrne et al. 1978; Walters et al. 1983a) and both function as wide-
dynamic-range nociceptors (see Walters, 1994). That is, they show graded responses to
mechanical stimulation, responding with one or two action potentials to relatively weak
stimuli, with brief bursts of action potentials to stronger stimuli, and with maximal
activation to intense, crushing stimuli which injure their receptive fields (Walters et al.
1983a; Clatworthy and Walters, 1993; P. A. Illich and E. T. Walters, unpublished
observations).

Noxious stimulation of the receptive field of a pleural VC sensory neurone causes not
only maximal activation of the cell but also particularly large and long-lasting
enhancement of the central and peripheral sensitivity of the sensory neurone and
enhancement of its synapses onto motor neurones (Walters, 1987a,b; Billy and Walters,
1989a,b). Although these effects were initially assumed to depend upon neuromodulators
released from facilitatory interneurones, a recent study suggests that peripheral injury of
sensory neurone branches may also directly trigger the same long-term reactions (Walters
et al. 1991). In this study synaptic facilitation and enhancement of soma excitability
lasting for weeks were produced by crushing nerves containing the axons of the tested
sensory neurones under conditions in which spike activity and neurotransmitter release
were severely reduced. Because there was a delay in the onset of sensory neurone
hyperexcitability related to the distance of the crush from the cell’s soma, Walters et al.
(1991) reasoned that some of the signals initiating injury-induced plasticity are conveyed
to the soma by retrograde axonal transport from the crush site. They suggested that this
form of plasticity represents a primitive adaptive response to neuronal injury which may
be particularly important in neurones, such as mechanosensory neurones, having
peripheral branches that are exposed to trauma.

The unexpected discovery that nerve crush produces robust plasticity of the VC
sensory neurone similar to that produced during aversive learning raised interesting
questions about the generality of this injury-induced plasticity and the nature of the
initiating signals. In the present paper, we have addressed three questions. First, do other
sensory populations in Aplysia also show long-term hyperexcitability following nerve
injury under conditions in which spike activity and neurotransmitter release play little or
no part? Second, is hyperexcitability induced by nerve injury restricted to sensory
neurones that evoke defensive reflexes, i.e. reflexes that would be expected to protect a
wounded region of the body? Third, is this plasticity specifically induced by crushing
axons of the tested sensory neurones, or can crush of nerves not containing axons of the
tested neurones also cause long-term hyperexcitability?

We have begun to answer these questions by systematically comparing the effects of
nerve crush on pleural VC sensory neurones and cerebral J and K sensory neurones
(hereafter termed the J/K neurones). The J/K mechanosensory neurones innervate the
head and mouth of the animal and have their somata in the cerebral ganglia. On the basis of their immunoreactivity to a sensory-neurone-specific peptide, the J/K neurones appear to be homologous with the LE, VC and several other centrally located mechanosensory populations in Aplysia and in other molluscs (Brunet et al. 1991; Montarolo et al. 1993; Steffensen et al. 1993). Nevertheless, the J/K neurones show interesting differences from the VC and LE neurones. Most importantly, their pharmacological properties and synaptic connections indicate that the J/K neurones (unlike the VC and LE neurones) are functionally heterogeneous, with some of them likely to have defensive roles and others to have a role in an appetitive behaviour – feeding (Rosen et al. 1979, 1982, 1989). Below we show that long-term hyperexcitability and synaptic facilitation following nerve crush occur in J/K sensory neurones as well as in VC sensory neurones, that this plasticity is not restricted to sensory neurones having a defensive function, and that the plasticity in both populations occurs only in sensory neurones with axons in injured nerves. Some of these results have been reported previously in abstract form (Clatworthy and Walters, 1991).

Materials and methods

Aplysia californica were obtained from Alacrity Marine Biological Services (Redondo Beach) and maintained in artificial sea water (ASW, Instant Ocean) at 17–19 °C. Surgery was performed after injecting ice-cold isotonic MgCl₂ solution (equivalent to about 30 % of body weight) with the animal suspended in a chamber containing ASW at 1–2 °C. These procedures block synaptic transmission, greatly reduce spike activity and eliminate the signs of behavioural sensitization (tonic immobility, suppression of feeding responses) that are often observed after surgery under less effective forms of anaesthesia (see also Walters et al. 1991). A small incision was made in the skin to expose the cerebral ganglia. Peripheral cerebral or pedal nerves containing axons of J/K or VC sensory neurones innervating one side of the body were crushed using fine (number 5) forceps at a distance of approximately 1 cm from the cerebral ganglion (Fig. 1) or pedal ganglion. Each crush caused apparent separation of the axons for a distance of about 0.5 mm within the nerve (as seen through the dissecting microscope), but did not sever the overlying sheath. The present experiments utilized nerve crush rather than nerve transection to damage the sensory neurone axons so that the results could be compared directly with those of a previous study (Walters et al. 1991) and with current studies of neuronal regeneration (Dulin and Walters, 1993), which involve nerve crush. The incision was sutured immediately after all the ipsilateral peripheral nerves from the cerebral or pedal ganglion had been crushed, and the animal was returned to its tank. Nearly all animals survived the surgery without apparent ill effect, despite the lack of sterile procedures or the use of antibiotics. One to ten days after surgery, the animal was anaesthetized by injection of a volume of isotonic MgCl₂ equivalent to approximately 50 % of its body weight. The ring ganglia were dissected out and the cerebral and/or the pleural ganglia were desheathed in a 1:1 mixture of ASW and isotonic MgCl₂ solution. The ganglia were then superfused with ASW at a constant rate for the duration of the experiment.

Sensory neurones were impaled with glass capillary microelectrodes (10–20 MΩ)
filled with 3 mol l$^{-1}$ potassium acetate. A series of J/K sensory neurones or VC sensory neurones was examined sequentially, alternating between right and left clusters, with each bilateral pair of cells matched by location in the sensory cluster. Spike threshold, amplitude, duration and the magnitude of the spike afterhyperpolarization (AHP) were recorded during responses to a brief (20 ms) intracellular depolarizing pulse (see Walters et al. 1991). Amplitude measurements were taken on spikes that began after the offset of the depolarizing test pulse. Spike duration was measured as the time from the peak to the first intersection with the extrapolated resting potential (measured immediately before the test pulse). As an index of effective soma excitability, we recorded the number of spikes evoked by a 1 s injection of current 25% greater than the threshold current determined with the 20 ms pulses. Input resistance was monitored by measuring the change in membrane potential during injection of 1 s, 0.5 nA hyperpolarizing pulses. In experiments examining alterations of J/K sensory neurone synapses, two postsynaptic motor neurones were recorded simultaneously, one in each B cluster (‘Bn’ neurones, identified by their narrow action potentials; Teyke et al. 1989; see also Fredman and Jahan-Parwar, 1977; Rosen et al. 1979). A series of sensory neurones was examined sequentially, alternating back and forth between each hemiganglion, and the magnitude of the excitatory postsynaptic potential (EPSP) in each Bn neurone produced by the initial spike in the sensory neurone was measured. The preparation was bathed in a solution containing three times the normal concentrations of Ca$^{2+}$ (i.e. 33 mmol l$^{-1}$) and Mg$^{2+}$ (i.e. 165 mmol l$^{-1}$) which, by raising the spike threshold of interposed interneurones, minimized the contribution of polysynaptic inputs to the synaptic potential (see Walters et al. 1983a). All EPSPs displayed a latency of 7 ms or less, putting them in the range previously reported for monosynaptic sensorimotor connections in Aplysia ganglia (e.g. Byrne et al. 1974; Rosen et al. 1979; Walters et al. 1983a).
Statistical comparisons between animals were made with two-tailed paired t-tests using mean scores per side (averaging data from 4–8 cells per side in each animal) as individual data points. Statistical significance was taken as \(P<0.05\).

**Results**

J/K cerebral sensory neurones send axons to the periphery via ipsilateral cerebral nerves (Rosen *et al.* 1979, 1982). Fig. 2 illustrates two major long-term effects on J/K neurones caused by the unilateral crush 6 days earlier of cerebral nerves containing axons of the tested sensory neurones. These effects are analyzed in detail in subsequent sections. First, it can be seen that the J/K sensory neurone having an axon in the crushed nerves was more excitable, responding to a 1 s pulse of depolarizing current (normalized to threshold) with more spikes than the paired J/K neurone on the contralateral side, whose axons were not damaged by the crush procedure. The shorter first interspike interval in the cell with the crushed axon indicates that this increase in excitability involves a decrease in spike accommodation. The brief, rapidly accommodating response of the contralateral J/K sensory neurone to sustained depolarization is typical of mechanosensory neurones that have been studied in *Aplysia* (e.g. Klein *et al.* 1986; Baxter and Byrne, 1990; Walters *et al.* 1991). The second major effect was facilitation of the synaptic connection from the J/K sensory neurone with an axon in the crushed nerves onto a cerebral Bn motor neurone; this is indicated by the greater amplitudes of the first two EPSPs on the crushed side than on the uncrushed side.

Fig. 2. Example of electrophysiological alterations observed in J/K sensory neurones 6 days after ipsilateral cerebral nerve crush. Intracellular recordings were made in the same preparation during the application of a 1 s depolarizing pulse (1.25 times the 20 ms threshold current) to each sensory neurone. A solution containing elevated (three times normal) Ca\(^{2+}\) and Mg\(^{2+}\) levels was used to reduce polysynaptic contributions to the observed synaptic potentials. (A) A Bn motor neurone and a J/K sensory neurone recorded in the cerebral hemiganglion contralateral to the crushed cerebral nerves; (B) corresponding cells in the opposite hemiganglion, ipsilateral to the crushed nerves. Both the sensory and motor neurone in B were likely to have had crushed axons, while the corresponding neurones in A would not have had crushed axons. Note the greater number of sensory neurone spikes and the larger amplitude of the first two EPSPs in the motor neurone on the crushed side compared with the uncrushed side. Action potential amplitudes have been attenuated by the limited frequency response of the pen recorder.
Cerebral nerve crush enhances the excitability of cerebral sensory neurone somata

Cerebral sensory neurones with crushed axons displayed alterations of several electrophysiological properties of the soma. The excitability of the J/K neurones was significantly elevated, as indicated by the larger number of spikes evoked by the 1 s depolarizing pulse injected into the soma of cells having crushed axons compared with contralateral cells with uncrushed axons (Fig. 2, Table 1). Because the intracellular test stimulus intensity was normalized to the cell’s spike threshold, this measure reflects a difference in repetitive firing properties (including a decrease in spike accommodation) rather than a decrease in threshold. The threshold for generating a spike with a 20 ms depolarizing pulse displayed a small but reliable decrease in cerebral sensory neurones with crushed axons (Table 1). The amplitude of the spike afterhyperpolarization (AHP) also showed a significant decrease, while spike duration was significantly increased. No significant differences between control neurones and neurones with crushed axons were observed in spike amplitude, input resistance or resting potential.

Walters et al. (1991) showed that hyperexcitability of pleural VC sensory neurones following axonal crush can take several days to be expressed, depending on the distance of the site of injury from the cell soma. We investigated whether crush-induced hyperexcitability followed a similar time course in cerebral J/K sensory neurones. Fig. 3 plots the relative excitability of cerebral sensory neurones as a function of time following unilateral crush of cerebral nerves approximately 1 cm from the J/K clusters. Each point represents the mean difference in spike number between ipsilateral and contralateral cerebral sensory neurones in 5–7 matched pairs of cells per animal. Cerebral sensory neurones with crushed axons fired slightly more spikes in response to the test stimulus than control cells in animals tested 1–3 days after the crush. In contrast,

<table>
<thead>
<tr>
<th>Property</th>
<th>Control</th>
<th>Crushed</th>
<th>P</th>
<th>N</th>
<th>Neurone pairs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Excitability (number of spikes per 1 s pulse)</td>
<td>1.9±0.1</td>
<td>4.6±0.3</td>
<td>0.0001</td>
<td>38</td>
<td>196</td>
</tr>
<tr>
<td>Threshold (nA)</td>
<td>1.1±0.1</td>
<td>0.9±0.1</td>
<td>0.0001</td>
<td>38</td>
<td>196</td>
</tr>
<tr>
<td>Duration (ms)</td>
<td>2.6±0.1</td>
<td>3.0±0.1</td>
<td>0.0004</td>
<td>26</td>
<td>116</td>
</tr>
<tr>
<td>Afterhyperpolarization (mV)</td>
<td>5.2±0.2</td>
<td>4.5±0.2</td>
<td>0.006</td>
<td>38</td>
<td>196</td>
</tr>
<tr>
<td>Amplitude (mV)</td>
<td>79.5±1.4</td>
<td>77.4±1.3</td>
<td>0.21</td>
<td>38</td>
<td>196</td>
</tr>
<tr>
<td>Input resistance (MΩ)</td>
<td>27.9±2.0</td>
<td>30.6±1.8</td>
<td>0.18</td>
<td>38</td>
<td>196</td>
</tr>
<tr>
<td>Resting potential (mV)</td>
<td>44.8±1.3</td>
<td>44.2±1.0</td>
<td>0.37</td>
<td>38</td>
<td>196</td>
</tr>
</tbody>
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Each value represents the mean ± s.e.m. of the average scores from J/K sensory neurones from each side of animals examined 4–10 days after unilateral cerebral nerve crush.

The P value was calculated using two-tailed, paired t-tests using the average scores per side of each animal as data points (N=26–38 animals). Each average score per side represents the mean of 4–8 ipsilateral cells in that animal. All neurones on the control and crushed sides were paired with each other by location and order of impalement. Thus, 116–196 cell pairs in 26–38 animals were used to make each comparison.

Cerebral nerve crush enhances the excitability of cerebral sensory neurone somata

Walters et al. (1991) showed that hyperexcitability of pleural VC sensory neurones following axonal crush can take several days to be expressed, depending on the distance of the site of injury from the cell soma. We investigated whether crush-induced hyperexcitability followed a similar time course in cerebral J/K sensory neurones. Fig. 3 plots the relative excitability of cerebral sensory neurones as a function of time following unilateral crush of cerebral nerves approximately 1 cm from the J/K clusters. Each point represents the mean difference in spike number between ipsilateral and contralateral cerebral sensory neurones in 5–7 matched pairs of cells per animal. Cerebral sensory neurones with crushed axons fired slightly more spikes in response to the test stimulus than control cells in animals tested 1–3 days after the crush. In contrast,
there was often a large increase in the number of spikes generated in cells with crushed axons 4–10 days after the nerve crush (see also Table 1). It is noteworthy that no animals showed greater average excitability on the control side than on the side with crushed nerves.

Cerebral nerve crush facilitates monosynaptic connections between cerebral sensory and motor neurones

Cerebral J/K neurones make monosynaptic connections to a subpopulation of neurones within the B clusters (Bn motor neurones) located in the cerebral ganglion (Teyke et al. 1989). We examined whether the amplitude of these connections was modified by axonal crush. To reduce the contribution of polysynaptic components to the measured EPSPs, a bathing solution containing elevated concentrations of Ca$^{2+}$ and Mg$^{2+}$ was used to increase spike threshold in interneurones excited by the J/K sensory neurones. Monosynaptic connections between cerebral sensory and motor neurones ipsilateral to the crushed cerebral nerves were compared with the corresponding connections in sensory and motor neurones contralateral to the crushed nerves. Ipsilateral sensory neurones would be expected to have a much larger number of crushed axons than contralateral sensory neurones (see Rosen et al. 1979). As shown in Fig. 4 (see also Fig. 2), J/K sensory neurones ipsilateral to the crushed nerves displayed significant enhancement of EPSP amplitude relative to the corresponding connections on the uncrushed side of the animal. The connections of ipsilateral sensory neurones (having crushed axons) to contralateral motor neurones were also larger than the connections of
contralateral sensory neurones (having uncrushed axons) to ipsilateral motor neurones (1.2 versus 0.9 mV; \( N \)=11 animals), but this difference was not significant. In addition, no significant alterations were found in the ipsilateral synaptic connections of J/K sensory neurones to Bn neurones following unilateral crush of the pedal nerves rather than the cerebral nerves. As discussed below, the absence of plasticity following injury of nerves lacking axons of the tested sensory neurone suggests that this plasticity may require damage to the axon of the tested neurone.

Cerebral nerve crush injures the axons of the Bn motor neurones as well as the axons of the J/K sensory neurones, which raises the possibility that injury-induced postsynaptic alterations may contribute to the synaptic facilitation following ipsilateral nerve crush. However, such contributions do not appear to involve a general increase in input resistance of the postsynaptic neurone, since no significant difference was found in input resistance of ipsilateral (9.5±1.8 M\( \Omega \)) and contralateral (10.9±1.6 M\( \Omega \)) Bn motor neurones following unilateral cerebral nerve crush.

Crush-induced sensory hyperexcitability is similar in cerebral and pleural sensory neurones

The alterations in cerebral J/K sensory neurones summarized in Table 1 are quite similar to those reported in pleural VC sensory neurones after axonal injury (Walters et al. 1991): both populations exhibited an increase in excitability and spike duration and a decrease in spike threshold and spike AHP following crush of ipsilateral nerves containing axons of the tested sensory neurones. However, there were also some apparent

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**Fig. 4.** Long-term facilitation of synaptic connections between cerebral J/K sensory neurones and Bn motor neurones 4–8 days after ipsilateral cerebral nerve crush. The mean amplitude of the initial EPSP evoked by each sensory neurone is shown. Measurements were made in sea water containing three times the normal levels of \( \text{Ca}^{2+} \) and \( \text{Mg}^{2+} \) (raising the threshold of interneurones and thus minimizing polysynaptic contributions). The asterisk indicates \( P<0.05 \) (paired \( t \)-test; \( N=11 \) animals). The EPSPs to a single Bn motor neurone in each hemiganglion from 5–10 different sensory neurones per hemiganglion were averaged in each animal. The error bars in this and subsequent figures represent the S.E.M.
quantitative differences between properties of the cerebral and pleural sensory neurones. In particular, the pleural sensory neurones showed greater excitability and a higher spike threshold in both the crushed and uncrushed conditions than the cerebral sensory neurones. These differences between the J/K and VC neurones may be misleading though, since the two studies were performed on different populations of animals at different times, and various behavioural and neuronal processes show seasonal variation in Aplysia (e.g. Kupfermann, 1970; E. T. Walters, unpublished observations). To make more direct comparisons between injury-induced plasticity in cerebral J/K sensory neurones and pleural VC sensory neurones, we performed simultaneous, unilateral crushes on both the cerebral and pedal nerves in the same animals (N=6). Under these conditions the electrophysiological alterations produced by nerve injury were almost identical in the J/K and VC sensory neurones (Fig. 5). Neurones from both sensory populations displayed significant increases in excitability and spike duration, and decreases in spike threshold, 5–8 days after crush of their axons. The degree of change in each of these properties was quite similar for each sensory population. In contrast to the findings of the larger studies reported in Table 1 and by Walters et al. (1991), no significant decrease in spike AHP was observed in this study following axonal injury of either the cerebral or the pleural sensory neurones.

**Crush-induced hyperexcitability is not restricted to sensory neurones with defensive functions**

To begin to determine whether crush-induced sensory hyperexcitability is an effect specific to sensory neurones having a defensive function or is also expressed in sensory neurones having other functions, we compared the incidence of crush-induced hyperexcitability in pleural and cerebral sensory populations. As explained in the Discussion, all of the pleural VC sensory neurones appear to have a defensive function, whereas about 50 % of the cerebral J/K neurones may be involved in appetitive rather than defensive behavior. If crush-induced sensory plasticity occurs in all Aplysia sensory neurones, regardless of function, there should be no significant difference in the incidence of long-term hyperexcitability in pleural and cerebral sensory populations following crush of all their axons. Conversely, if crush-induced sensory plasticity is specific to sensory neurones having a defensive function, we would predict that the incidence of cerebral sensory hyperexcitability would be less than that recorded in pleural sensory neurones after crush of all their axons. We initially examined data from experiments in which either unilateral pedal or cerebral nerve crush was performed. The incidence of hyperexcitability was measured as the percentage of neurones on the side with crushed nerves that fired more spikes in response to the 1 s depolarizing test pulse than the mean number of spikes evoked in all tested sensory neurones on the uncrushed side by the same test stimulus in the same animal. Three to eight days after nerve crush, 91 % of pleural VC sensory neurones (32 of 35 cells) having axons in crushed nerves were hyperexcitable by this index, while only 67 % of cerebral J/K sensory neurones (29 of 46 cells) were hyperexcitable (Fig. 6A,B). One limitation of this comparison, however, is that approximately 50 % of the cerebral J/K neurones have axons in the ipsilateral cerebral–pleural or cerebral–pedal connectives (Rosen et al. 1979). Thus, an
Fig. 5. Comparison of long-term hyperexcitability induced simultaneously in cerebral J/K and pleural VC sensory neurones (SNs) in the same animals. Crushed nerves are marked by crosses and the axons of the recorded sensory neurones by dark lines. Control data were obtained from sensory neurones contralateral to the crushed nerves, while crushed data were obtained from sensory neurones ipsilateral to the crushed nerves. Asterisks indicate $P<0.05$ ($N=6$ animals). Data from 5–7 sensory neurones were averaged on each side in each animal. AHP, afterhyperpolarization.
unknown but substantial number of J/K neurones may not have sustained axonal damage when the crushes were applied solely to peripheral nerves. In contrast, all the axons of the pleural VC sensory neurones appear to enter ipsilateral pedal nerves (Walters et al. 1983a, b; E. T. Walters and A. J. Billy, unpublished observations). When we examined preparations in which the ipsilateral cerebral–pleural and cerebral–pedal connectives had been crushed in addition to the ipsilateral cerebral nerves (thereby crushing all the ipsilateral J/K sensory neurone axons), the incidence of hyperexcitability in cerebral J/K sensory neurones (93 %, that is 42 of 45 cells) was almost identical to the incidence of hyperexcitability in pleural VC sensory neurones after ipsilateral pedal nerve crush (Fig. 6C). The large fraction of sensory neurones that became hyperexcitable in each case suggests that the hyperexcitability may occur in all centrally located mechanosensory neurones whose axons are crushed, regardless of the function of the mechanosensory neurones. Furthermore, the increase in frequency of hyperexcitability in J/K neurones when the ipsilateral cerebral–pedal and cerebral–pleural connectives were crushed (in addition to the cerebral nerves) suggests that injury to central connectives, as in peripheral nerves, can produce hyperexcitability if the connectives contain axons of the
tested sensory neurones (see also Walters et al. 1991, for similar results in the VC neurones when the pleural–pedal connective was crushed). Thus, injury-dependent induction signals for long-term soma hyperexcitability are not restricted to peripheral nerves.

**Crush-induced hyperexcitability is specific to sensory neurones with damaged axons**

Walters et al. (1991) concluded that axonal injury may be sufficient to produce long-term hyperexcitability in *Aplysia* sensory neurones. An essential part of their argument rested on the presumed specificity of this plasticity to sensory neurones having axons in injured nerves. Here we present evidence for this specificity by showing that long-term hyperexcitability of the sensory neurone soma is not produced by crushing nerves lacking axons of the tested neurones. Fig. 7 shows the results of experiments similar to those summarized in Fig. 5 except that only the pedal or cerebral nerves were crushed and the tested sensory neurones were in clusters that were subject to little if any axonal injury in the crushed nerves. It can be seen (i) that crushing the ipsilateral pedal nerves failed to alter cerebral sensory neurone properties significantly and (ii) that crushing the ipsilateral cerebral nerves failed to alter pleural sensory neurone properties significantly (N=8 in each case). These results show that massive nerve damage relatively distant from a tested sensory neurone is not sufficient to produce long-term hyperexcitability in that neurone if it does not have an axon in the damaged nerves. However, potential non-axonal plasticity signals released during nerve injury might have a limited spatial spread. To determine whether nearby nerve injury (in the absence of damage to the axons of the tested sensory neurones) could cause long-term hyperexcitability of sensory neurone somata, we took advantage of the anatomy of the VC sensory neurones. The VC cluster shows a rough somatotopic organization in which sensory neurones innervating the tail region have their somata in the caudal-dorsal part of the cluster (Walters et al. 1983a), while none of the cells in this part of the cluster have been observed to send axons into the cerebral–pleural or pleural–abdominal connectives (E. T. Walters and A. J. Billy, unpublished observations; M. F. Dulin, I. Steffensen, C. E. Morris and E. T. Walters, unpublished observations). Fig. 8 shows long-term (3–4 days) effects on soma excitability in caudal VC neurones in a study in which all the pedal nerves were crushed 2 cm from one pedal ganglion, while both the cerebral–pleural and pleural–abdominal connectives were crushed less than 1 mm from the contralateral pleural ganglion. This procedure caused the VC sensory neurones on one side of the animal to be exposed to the effects of relatively distant peripheral nerve injury that damaged all the axons of the tested sensory neurone, while sensory neurones on the other side were exposed to potential effects of relatively close neural connective injury that did not damage the axons of the tested sensory neurones. It can be seen that the sensory neurones whose axons were injured showed significantly greater excitability than the sensory neurones that were close to the sites of connective crush but did not sustain damage to their axons. The latter showed relatively little excitability (compare Figs 5 and 8) and showed no statistically significant differences from sensory neurones contralateral to unilateral pedal nerve crush in animals examined during the same period.
The present studies demonstrate that similar, long-term neuronal plasticity produced by nerve crush occurs in functionally diverse mechanosensory neurones of Aplysia. This plasticity takes the form of hyperexcitability and synaptic facilitation in sensory neurones

Discussion

The present studies demonstrate that similar, long-term neuronal plasticity produced by nerve crush occurs in functionally diverse mechanosensory neurones of Aplysia. This plasticity takes the form of hyperexcitability and synaptic facilitation in sensory neurones.
suffering axonal damage. The alternative possibility (that nerve injury depresses excitability and synaptic transmission in sensory neurones contralateral to the injury) is highly unlikely because the properties of pleural and cerebral sensory neurones with uncrushed axons were indistinguishable from those of uncrushed pleural sensory neurones of sham-operated animals examined in a concurrent study (Clatworthy et al. 1994) or in naive animals (A. L. Clatworthy and E. T. Walters, unpublished observations). The present studies also provide the first evidence that delayed, injury-induced plasticity is specific to sensory neurones with axons in the crushed nerves. As discussed below, this axonal specificity restricts the set of potential signals that trigger and maintain long-term sensory plasticity after severe peripheral injury.

**Similarity of injury-related alterations in cerebral and pleural sensory neurones**

Previously, Walters et al. (1991) found that crush of all the nerves containing the peripheral axons of pleural VC sensory neurones caused a delayed, persistent
hyperexcitability of the soma (involving an increase in soma excitability and spike duration, and a decrease in spike threshold and afterhyperpolarization) as well as long-lasting facilitation of sensory neurone synapses. We have now found that the same alterations occur in the cerebral J/K sensory neurones and their ipsilateral synapses (Table 1, Fig. 5). Although we did not observe statistically significant alterations in the J/K neurones’ connections to contralateral Bn neurones, a larger sample size needs to be examined before firm conclusions can be made about the presence or absence of injury-induced facilitation of these more distant synapses of the J/K sensory neurones. A recent finding by Dulin and Walters (1992) indicates that a detailed analysis of the mechanisms of synaptic facilitation induced by nerve injury will be complicated; they observed that the excitability of pedal motor neurones increases after their axons are crushed. Interestingly, motor neurone input resistance failed to change in either that study or the present one. The unexpected discovery of delayed motor neurone hyperexcitability suggests that synaptic facilitation observed after nerve injury may involve postsynaptic as well as presynaptic alterations.

The similarity in the pattern of electrophysiological alterations observed in the sensory neurone soma following axonal injury of cerebral J/K neurones and pleural VC neurones (Walters et al. 1991) was confirmed with direct comparisons of alterations in these two sensory populations using identical methods in the same animals. The similarity was even evident in the weaker expression of the altered AHP compared with the other alterations. While a significant reduction of the AHP in each sensory population has been found in two studies (Table 1 and Walters et al. 1991), a statistically significant reduction in AHP was not observed in either the cerebral J/K or pleural VC sensory neurones in the study summarized in Fig. 5, even though both populations showed significant changes in excitability, spike threshold and spike duration. The lack of a clear effect on the AHP probably reflects the smaller sample size of this study (N=6 animals) compared with the earlier cerebral sensory neurone study (N=38; Table 1) and pleural sensory neurone study (N=26; Walters et al. 1991). It is interesting that reduction of the sensory neurone AHP has also been more difficult to observe in studies of the effects of learning or learning-related signals on Aplysia sensory neurones. Such a reduction has only been mentioned in one report (Klein et al. 1986), whereas spike broadening, synaptic facilitation and increases in spike threshold and accommodation (increased excitability) have been quite prominent in many studies of learning-related alterations in these cells (see reviews by Byrne, 1987; Hawkins et al. 1993; Walters, 1994). Similarities between injury-induced plasticity and learning-related plasticity in two different sensory populations in Aplysia suggest that axonal injury and sensory memories underlying defensive sensitization and aversive classical conditioning may depend upon a common set of cellular alterations.

We do not yet know the conductance alterations that underlie long-term sensory neurone hyperexcitability following nerve crush, but one possibility is that there is persistent depression of the same K+ currents that have been observed to be depressed in Aplysia sensory neurones over much shorter periods following cutaneous shock or 5-HT application. These currents include (1) the S-type K+ current which is partially active at the resting potential (e.g. Klein et al. 1982; Scholz and Byrne, 1987; Baxter and Byrne, 1989; Goldsmith and Abrams, 1992); (2) a transient, highly voltage-dependent ‘delayed
rectifier $K^+$ current (Baxter and Byrne, 1989; Goldsmith and Abrams, 1992; Hochner and Kandel, 1992); and (3) a steady-state $Ca^{2+}$-activated $K^+$ current (Walsh and Byrne, 1989). It is interesting that axotomy of some mammalian sensory and sympathetic neurones is reported to produce increased soma excitability, spike broadening and a reduction of the AHP, and it has been suggested that these effects reflect a depression of $K^+$ conductances, including a $Ca^{2+}$-dependent $K^+$ conductance (e.g. Gallego et al. 1987; Gurtu and Smith, 1988; see Titmus and Faber, 1990, for a review). However, the most commonly suggested mechanism underlying soma hyperexcitability following axotomy in various vertebrate and invertebrate neurones has been an increase in the density of voltage-sensitive $Na^+$ channels (e.g. Goodman and Heitler, 1979; Kuwada and Wine, 1981; Roederer and Cohen, 1983; Sernagor et al. 1986; Titmus and Faber, 1986). The relative contributions of these and other potential ionic mechanisms (e.g. increased $Ca^{2+}$ conductance) to injury-induced soma hyperexcitability in *Aplysia* sensory neurones will be amenable to analysis in these sensory neurones.

Sensory neurones with diverse functions display hyperexcitability after nerve injury

All of the sensory neurones in the pleural VC clusters appear to be involved in triggering defensive responses (Walters et al. 1983a; E. T. Walters, unpublished observations). By contrast, in the cerebral J/K population some sensory neurones seem to be involved in defensive behaviour (Rosen et al. 1989; Teyke et al. 1989) and others in feeding (Rosen et al. 1982). The relative proportions of each type are not yet known, but clues may be available from two patterns of response within the J/K neurones to the neuromodulators 5-HT and FMRFamide. 5-HT is necessary for behavioural sensitization of gill responses under some conditions (Glanzman et al. 1989) and has been observed to sensitize tail withdrawal responses (Walters et al. 1983b). 5-HT enhances both the sensitivity and the synaptic output of the VC and LE sensory neurones (Klein and Kandel, 1978; Walters et al. 1983b; Klein et al. 1986; Baxter and Byrne, 1990; Billy and Walters, 1989b), while the neuropeptide FMRFamide has the opposite effects on these cells (Belardetti et al. 1987; Mackey et al. 1987; Billy and Walters, 1989b; Pieroni and Byrne, 1992). About 50% of the J/K sensory neurones resemble the VC and LE sensory neurones (which have defensive functions) by showing spike broadening responses to 5-HT and spike narrowing responses to FMRFamide (Rosen et al. 1989). Interestingly, about 50% of the J/K cells (including the ‘ICBM’ sensory neurones implicated in feeding behaviour; Rosen et al. 1989) show the opposite pattern: spike narrowing in response to 5-HT and spike broadening in response to FMRFamide. These observations led Rosen et al. (1989) to propose that the cells whose spikes are broadened by 5-HT have defensive functions while those that show spike narrowing in response to 5-HT participate in feeding behaviour. If this proposal is correct, and if individual sensory neurones evoke either defensive or appetitive responses but not both, about 50% of the J/K neurones have defensive functions and 50% have appetitive functions. Given this argument, it is interesting that there was no difference in the incidence of hyperexcitability following systematic crush of the nerves containing virtually all of the axons of the VC and J/K cells; in both populations about 90% of the cells with crushed axons were more excitable than the
average cell on the control side (Fig. 8). This suggests that long-term plasticity induced by nerve injury is not restricted to sensory neurones having defensive functions. Indeed, the present results, combined with the recent discovery that motor neurones also display long-term hyperexcitability following injury to the nerves containing their axons (Dulin and Walters, 1992), suggest that axonal injury causes long-term enhancement of neuronal signalling in many types of neurones in *Aplysia*. It will be interesting to see whether these responses represent a general adaptive reaction of neurones to bodily injury, and whether this reaction shows its greatest development in cells, such as sensory and motor neurones, having axons that project to the relatively vulnerable periphery of this soft-bodied animal.

**Functional model of injury-related sensory alterations**

The changes we have observed in this study are likely to correspond to changes that, under natural conditions, occur after damage to peripheral branches of a sensory neurone caused by body wall trauma (e.g. the bite of a predator; see Walters *et al.* 1993). Normally silent, a traumatized sensory neurone reacts to peripheral injury with an immediate, intense burst of spikes (Fig. 9). The sensory neurone then increases both its peripheral and central excitability and strengthens its synaptic connections to follower neurones. The resulting increase in signalling effectiveness presumably helps to protect (sensitize) the wounded site and to compensate for loss of sensory innervation of the site (Walters, 1987a; Billy and Walters, 1989a; Clatworthy and Walters, 1993; Walters, 1991, 1994). Slow, retrograde signals are produced at sites of nerve injury and travel to the nucleus (Fig. 9, and see below). These would be expected to contribute to the long-lasting maintenance of hyperexcitability and synaptic facilitation triggered initially by fast signals such as spike activity and neuromodulator release. The increase in central excitability of the sensory neurone described here and in previous studies would be expected to increase the likelihood of central afterdischarge in response to subsequent cutaneous stimuli (Clatworthy and Walters, 1993), while the increase in spike duration would be expected to enhance spike propagation through regions of the neurone having a low safety factor for conduction (Clatworthy and Walters, 1993) and to facilitate release of neurotransmitter from presynaptic terminals (e.g. Klein and Kandel, 1978; Hochner *et al.* 1986; Blumenfeld *et al.* 1990).

**What triggers the slow axonal injury signals?**

The failure to produce general hyperexcitability after unilateral crush of pedal nerves, cerebral nerves or pleural connectives suggests that the observed plasticity was not produced by a delayed activation of the modulatory interneurones (Fig. 9) that are thought to mediate similar plasticity during behavioural sensitization and classical conditioning (Hawkins *et al.* 1981; Mackey *et al.* 1989). Activation of modulatory interneurones during nerve crush was highly unlikely because synaptic transmission and axonal conduction had been severely reduced by cooling and high Mg²⁺ levels. However, the possibility remained that, following recovery from anaesthesia, facilitatory interneurones could have become activated by after effects of the injury. Arguing against this possibility is the topographic specificity of the plasticity produced by unilateral nerve
crush, which contrasts with the generality of sensitizing effects thought to be mediated by nociceptive activation of modulatory interneurones. In particular, enhancement of withdrawal responses, escape locomotion and sensorimotor synapses following cutaneous shock or nerve shock often involves both sides as well as both the anterior and posterior ends of the animal (Pinsker et al. 1973; Walters, 1980, 1987a,b; Walters and Byrne, 1985; E. T. Walters, unpublished observations). This difference between the effects of nerve crush and noxious cutaneous or nerve stimulation suggests that long-term plasticity following nerve injury involves a set of signals whose effects are restricted to neurones having axons within the injured nerve.

Fig. 9. Six potential types of signal for triggering long-term plasticity in Aplysia sensory neurones following peripheral injury. Traumatic injury to the receptive field of a sensory neurone initiates spike activity (including afterdischarge in the soma) and the release of chemical modulators from interneurones and perhaps other sources. The interaction of spike activity and modulators (ADEM) can rapidly lead to hyperexcitability and synaptic facilitation (both of which increase signalling strength, indicated here by thickened lines). In the soma, these rapid signals may alter gene expression and protein synthesis, thereby initiating similar long-term alterations as well as regenerative and collateral sprouting. Slow signals may be generated at sites of neuronal injury in response to one or more consequences of local damage and travel via retrograde axonal transport to the soma in order to maintain and perhaps amplify the effects already initiated by the rapid, activity-dependent signals. Neuronal growth occurs both centrally and peripherally, increasing the number of presynaptic terminals and the size of the peripheral arborization. In the present study it is likely that only the slow signals were available to produce the observed alterations.

• Activity-dependent extrinsic modulation (ADEM)
• Afterdischarge

• Hyperexcitability
• † Transmitter release

• Hyperexcitability
• † Transmitter release
• Central sprouting
• Peripheral sprouting
As observed previously in the VC sensory neurones (Walters et al. 1991), pronounced hyperexcitability of the J/K somata was not observed until several days after ipsilateral nerve crush (Fig. 3). This observation, coupled with the observation that fast, activity-dependent signals are almost completely blocked during the anaesthesia procedure we used during nerve crush (see Walters et al. 1991), suggests that in each sensory population the induction signals travel slowly from the sites of injury to the soma by retrograde axonal transport (Fig. 9). The long delay could also have other causes; for example, injury signals may slowly build up to a threshold level. Additional evidence for injury signals being conveyed by retrograde axonal transport is provided by our finding that hyperexcitability after nerve crush occurred only in sensory populations that had sustained axonal injury; it was not observed in undamaged sensory neurones contralateral (Table 1, Fig. 5) or ipsilateral (Figs 7, 8) to sensory neurones whose axons had been crushed. This indicates that either axonal injury itself or the injury to cells close to the sensory neurone axon is responsible for triggering long-term plasticity. The failure to observe long-term hyperexcitability when connectives were crushed only 1–2 mm from the pleural VC somata (Fig. 8) indicates that, if the trigger for long-term plasticity originates in other cells, these cells must be very close to the affected axons. Nearby signals could come from substances released from other injured axons in the crushed nerve, from crushed glia or connective tissue, or from immunocytes attracted to the crush site (Fig. 9). A potential role for immunocytes in triggering the observed alterations is suggested by the finding that experimental induction of a host defence reaction near sensory neurone axons (by loosely ligating cotton fibre around peripheral nerves) causes similar long-term hyperexcitability of the VC sensory neurone somata which is associated with an accumulation of amoebocytes around the nerve (Clatworthy and Walters, 1992; Clatworthy et al. 1993, 1994).

Another potential trigger for long-term, injury-induced plasticity is a negative one: interruption of retrograde transport of trophic signals that are normally provided by peripheral target and satellite cells. Several lines of evidence suggest that continuing exposure to peripheral trophic signals such as nerve growth factor (NGF) is important for maintaining differentiated electrophysiological properties of various vertebrate neurones, and that removal of NGF can produce effects similar to those produced by axotomy (e.g. Purves, 1988; Titmus and Faber, 1990; but see Traynor et al. 1992). Interestingly, a recent study suggested that an NGF-like factor may exist in molluscs (Ridgway et al. 1991). However, positive signals conveyed by retrograde axonal transport following axotomy have also been implicated in mammals (Singer et al. 1982), and in Aplysia a molecular pathway for such signals (involving the injury-induced transport of specific proteins from the periphery to the nucleus) is being characterized (Ambron et al. 1992; Schmied et al. 1993). Identified populations of mechanosensory neurones in Aplysia should prove useful for examining the relative contributions of these and other potential signals to long-term neuronal plasticity induced by injury.

The authors are grateful to M. Dulin for helpful comments and J. Pastore for producing the illustrations. This work was supported by grants MH38726 from the NIMH and BNS-9011907 and IBN-9210268 from the NSF.
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