Long-Term Effects of Axotomy on Excitability and Growth of Isolated Aplysia Sensory Neurons in Cell Culture: Potential Role of cAMP

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Bedi, Supinder S., Ali Salim, Shanping Chen, and David L. Glanzman. Long-term effects of axotomy on excitability and growth of isolated Aplysia sensory neurons in cell culture: potential role of cAMP. J. Neurophysiol. 79: 1371–1383, 1998. Crushing nerves, which contain the axons of central sensory neurons, in Aplysia causes the neurons to become hyperexcitable and to sprout new processes. Previous experiments that examined the effects of axonal injury on Aplysia sensory neurons have been performed in the intact animal or in the semi-intact CNS of Aplysia. It therefore has been unclear to what extent the long-term neuronal consequences of injury are due to intrinsic or extrinsic cellular signals. To determine whether injury-induced changes in Aplysia sensory neurons are due to intrinsic or extrinsic signals, we have developed an in vitro model of axonal injury. Isolated central sensory neurons grown for 2 days in cell culture were axotomized. Approximately 24 h after axotomy, sensory neurons exhibited a greater excitability—reflected, in part, as a significant reduction in spike accommodation—and greater neuritic outgrowth than did control (unaxonotomized) neurons. Rp diastereoisomer of the cyclic adenosine 3′,5′-monophosphorothionate (Rp-cAMPS), an inhibitor of protein kinase A, blocked both the reduction in accommodation and increased neuritic outgrowth induced by axotomy. Rp-cAMPS also blocked similar, albeit smaller, alterations observed in control sensory neurons during the 24-h period of our experiments. These results indicate that axonal injury elevates cAMP levels within Aplysia sensory neurons, and that this elevation is directly responsible, in part, for the previously described long-term electrophysiological and morphological changes induced in Aplysia sensory neurons by nerve crush. In addition, the results indicate that control sensory neurons in culture are also undergoing injury-related electrophysiological and structural changes, probably due to cellular processes triggered when the neurons are axotomized during cell culturing. Finally, the results provide support for the idea that the cellular processes activated within Aplysia sensory neurons by injury, and those activated during long-term behavioral sensitization, overlap significantly.

INTRODUCTION

Injury of central mechanoreceptive sensory neurons, which mediate defensive withdrawal reflexes of the marine mollusk Aplysia californica, induces several long-term electrophysiological alterations in the cells. Thus crushing nerves, which contain the axons of the sensory neurons, causes these neurons to become hyperexcitable, broadens their action potentials, and induces long-term facilitation of transmitter release from their terminals (Gunstream et al. 1995; Walters et al. 1991). In addition to these electrophysiological changes, axonal injury triggers morphological changes in Aplysia sensory neurons. Specifically, after damage to the peripheral processes of tail sensory neurons, the cells bodies of which lie in the central pleural ganglia, processes of the neurons can be observed in regions of the CNS to which they ordinarily never project (Steifensen et al. 1995). This result indicates that peripheral axonal injury causes central sprouting of Aplysia sensory neurons. The electrophysiological effects of axonal injury on sensory neurons begin to appear within ~10 h (Gunstream et al. 1995; Walters et al. 1991), and both the electrophysiological and morphological effects persist for weeks after nerve crush.

The long-term electrophysiological and structural changes that take place in Aplysia sensory neurons after axonal injury are strikingly reminiscent of those that occur in these cells during long-term behavioral sensitization. This form of non-associative learning is induced by repeated presentation of noxious stimuli, such as electrical shocks to the animal’s head or tail (Frost et al. 1985; Pinsker et al. 1973). Stimuli that induce long-term behavioral sensitization in Aplysia have been shown to result in long-term reduction of the S-type K+ current, which is thought to enhance excitability of the sensory neurons (Scholz and Byrne 1987; see also Byrne and Kandel 1996); long-term enhancement of transmission at sensorimotor synapses (Frost et al. 1985); and a long-term increase in the number of branches and varicosities on the neurites of sensory neurons (Bailey and Chen 1983, 1988).

There is considerable understanding of the cellular and molecular bases of behavioral sensitization in Aplysia. Thus it is known that sensitizing stimuli activate serotonergic facilitatory interneurons within the CNS of Aplysia (Glanzman et al. 1989; Mackey et al. 1989). Serotonin (5-HT), released from the terminals of these interneurons, binds to receptors on sensory neurons (Kistler et al. 1985; Zhang et al. 1991), thereby causing an elevation in adenosine 3′,5′-cyclic monophosphate (cAMP) within these neurons (Bernier et al. 1982; Ocorr et al. 1986). The increased levels of cAMP within the sensory neurons enhance evoked release of transmitter from the sensory neuron terminals, producing facilitation of synapses between the sensory neurons and their follower cells (Brunelli et al. 1976; Ghirardi et al. 1992; Klein 1993). This presynaptic facilitation of the sensorimotor synapses contributes to sensitization of the withdrawal reflexes. An intracellular rise in cAMP has additional physiological actions in Aplysia sensory neurons that are believed to contribute to behavioral sensitization. Thus increased levels of cAMP reduce K+ currents within sensory neurons (Goldsmith and Abrams 1992; Hochner and Kandel 1992; Scholz and Byrne 1988), broaden the action potential of sensory neurons (Baxter and Byrne 1990; Klein 1993; Klein and Kandel 1978; although see Klein 1994), and induce hyperexcitability of sensory neurons, reflected both as a reduction
in spike accommodation to prolonged current pulses (see further) and a decrease in spike threshold (Baxter and Byrne 1990; Klein et al. 1986). Furthermore, just as repeated application of painful or noxious stimulation leads to long-term behavioral sensitization (Frost et al. 1985), repeated or prolonged application of either 5-HT or cAMP induce persistent electrophysiological and morphological changes in *Aplysia* sensory neurons that mimic those observed during long-term sensitization. Among these changes are a reduction of \( K^+ \) current (Scholz and Byrne 1988), reduction in accommodation (Dale et al. 1987), increased presynaptic release (Dale et al. 1988; Montarolo et al. 1986; Schacher et al. 1988), and structural remodeling (Glanzman et al. 1990; Nazif et al. 1991; O’Leary et al. 1995; Schacher et al. 1988).

The similarity between the effects on *Aplysia* sensory neurons of injury and of stimuli that induce long-term sensitization has led to the proposal that injury and sensitization-inducing stimuli trigger some of the same intracellular signals within the sensory neurons (Walters and Ambron 1995). However, the evidence that injury precipitates the same cellular changes as do sensitizing stimuli has come predominately from experiments performed on either intact *Aplysia* or isolated ganglia maintained in organ culture. This raises the question of to what extent the effects of nerve crush are actually due to cellular signals intrinsic to the sensory neurons. It is possible that these effects are due, instead, to extrinsic signals, such as neurotransmitters (or other substances) released by damaged nonsensory neurons the axons of which also run in the crushed nerves; to factors released from glial or other support cells; or —in the case of experiments on intact *Aplysia*—to a neuroimmune response produced by tissue damage. Indeed, many of the physiological effects of nerve crush on sensory neurons can be mimicked simply by loosely ligating nerves that contain the axons of sensory neurons, a manipulation that neither damages the axons nor disrupts their ability to conduct action potentials (Clatworthy et al. 1994). Furthermore, molluscan hemocytes—cells that mediate the immune response in mollusks—have been reported to contain 5-HT together with the cellular machinery for its rapid release (Stefano et al. 1989). It is therefore difficult, if not impossible, to disentangle the potential contribution of intrinsic, injury-induced signals from extrinsic signals—such as foreign body reactions of the nervous system—in whole-animal or organ-culture preparations.

To address the question of whether the effects of nerve crush on sensory neurons of *Aplysia* are due to intrinsic signals, we have developed a cell culture model of axonal injury. In addition, we have exploited this in vitro model system to test the hypothesis that at least some of the electrophysiological and morphological effects of injury are due to a rise of cAMP and activation of protein kinase A (PKA). Some of our results have been published previously in abstract form (Bedi and Glanzman 1996; Salim and Glanzman 1995).

**Methods**

**Cell cultures**

All of the cell cultures in our study consisted of isolated mechanosensory neurons that had been removed from ventrocaudal clusters of pleural ganglia of *A. californica* (Walters et al. 1983). The cell culture methods have been described previously (Rayport and Schacher 1986; Schacher and Proshansky 1983). The sensory neurons were dissociated individually and placed into cell culture far apart from one another so that their processes did not touch. Occasionally, we observed glia cells in some of our cell cultures. Such cultures were discarded; all of the cultures used for these experiments consisted entirely of isolated sensory neurons. The cultures were housed in a large capacity incubator (model 3919, Forma Scientific, Marietta, OH) at 18° for 2 d before the start of the experiments. After the completion of electrophysiological testing on day 1, the cell cultures were placed back into hemolymph-containing culture medium and returned to the incubator until testing on the following day. Some of the sensory neurons were axotomized before being returned to the incubator (see further).

**Electrophysiology and axotomy**

The experiments were performed at room temperature (20–22°). Before the start of each recording session, the hemolymph-containing culture medium was washed out of the culture dish and replaced with perfusion medium (50% sterile artificial seawater and 50% sterile Liebowitz-15 (L-15, Sigma, St. Louis, MO) plus appropriate salts. This medium was perfused through the culture dish at 0.4 ml/min. We intracellularly stimulated and recorded from the sensory neurons using standard techniques (Lin and Glanzman 1994b). The electrophysiological data were recorded and digitized with a MacLab 2e system (ADInstruments, Castle Hill, Australia). Before testing a neuron’s accommodation to prolonged pulses of constant current, we measured its input resistance and spike threshold. The input resistance was measured by injecting brief pulses of 0.1 nA negative current into the sensory neuron. Spike threshold was determined by injecting the sensory neuron with a graded series of 30-ms pulses of positive current. The single action potential triggered in the threshold measurement was used for the measurement of spike duration; spike duration was measured as the time between the peak of the action potential and the return of the membrane potential to baseline. After measuring a sensory neuron’s input resistance and spike threshold, we tested its accommodation. This was done by injecting the neuron with 2-s pulses of various levels of positive current; the current levels were 0.5–4 nA. After the electrophysiological tests of accommodation had been performed, some sensory neurons were axotomized with a glass microneedle. We cut through the major neurite of each cell approximately halfway down its length, and the severed distal portion of the neurite then was removed from the culture dish. Approximately 24 h later, the sensory neurons were reimpaled and their electrophysiological properties reassessed.

**Application of PKA inhibitor**

In the experiments testing the effects of PKA inhibition, Rp diastereoisomer of the cyclic adenosine 3',5'-monophosphorothiate (Rp-cAMPS, RBI) was first dissolved in 250 µl of L-15 and then pipetted into the cell culture dishes in a final concentration of 500 µM in the hemolymph-containing culture medium. This was done after the electrophysiological tests, just before returning the cell cultures to the incubator. In the axotomy experiments, the Rp-cAMPS was added to the cell culture dishes 2–3 min before the axons of the sensory neurons were severed. The cells remained in the Rp-cAMPS-containing medium until the start of electrophysiological testing on day 2 of the experiments.

**Assessment of morphology**

To determine the structural complexity of a sensory neuron, we photographed its entire outgrowth on days 1 and 2 with a charge-
coupled device (CCD) camera (Hamamatsu Photonics, Oak Brook, IL). The complexity of the neuron’s structure was assessed by comparing the number of branch points on day 1 with those on day 2. If a neuron had only a single main neurite with no branch points, the neuron was assigned a branch point value of 1.0.

**Statistics**

Within group statistical comparisons (day 1 vs. day 2) were made with paired t-tests. Statistical comparisons between two different experimental groups were made with nonparametric Mann-Whitney tests, as stated in the text. All indicated levels of statistical significance represent two-tailed values, unless otherwise indicated.

**Results**

Axotomy causes a long-term reduction in spike accommodation of isolated Aplysia sensory neurons in cell culture

In the first set of experiments, we tested the effect of axotomy on the spike accommodation of isolated sensory neurons in culture. When stimulated with prolonged pulses of positive current, Aplysia sensory neurons typically accommodate—i.e., they fire only at the beginning of the current pulse (Baxter and Byrne 1990; Klein et al. 1986). After their accommodation had been assessed, some of the neurons (axotomized) in the culture dish were axotomized. Nothing was done to the other (control) sensory neurons in the dish. When tested on the second experimental day, control neurons (n = 17) exhibited a modest, albeit significant, decrease in spike accommodation. Collapsed across all the current levels tested, the mean number of spikes rose from 6.9 ± 0.8 on day 1 to 8.4 ± 1.0 on day 2 (P < 0.01; Fig. 1, A and D). The axotomized sensory neurons (n = 11) exhibited a greater decrease in accommodation than did the controls, however. The mean number of spikes in axotomized neurons, collapsed across all current levels, was 6.0 ± 0.9 on day 1 and 17.0 ± 1.5 on day 2 (P < 0.0001 for the day 2 – day 1 difference; Fig. 1, B and E). The day 2 – day 1 difference score for the axotomized group (11.0 ± 1.5) was significantly greater than that for the control group (1.5 ± 0.6) (P < 0.0001, Mann-Whitney test; Fig. 1C). There were no significant differences between the control and axotomized cells with respect to the number of spikes evoked by the various levels of injected current on day 1 (P ≥ 0.3, Mann-Whitney tests). Severing the axons of isolated sensory neurons in dissociated cell culture therefore results in intrinsic intracellular signals that trigger a long-term enhancement in the excitability of the neurons.

Axotomy induces other long-term electrophysiological changes in cultured sensory neurons in culture and increases outgrowth of neurites

In a second series of experiments, we examined axotomy’s effect not only on spike accommodation but also on other properties of sensory neurons that are altered by 5-HT or cAMP (see Introduction). Specifically, we also quantified changes in input resistance, spike threshold, spike duration, and neurite outgrowth after axotomy. In our second set of experiments, both control and axotomized cells exhibited significant decreases in spike accommodation on the second day of the experiment. (In this set of experiments, we tested the accommodation of sensory neurons with just 1 nA of current rather than multiple levels of current.) Thus the mean number of action potentials fired by control cells (n = 14) in response to a 2-s pulse of 1 nA current was 3.9 ± 1.0 on day 1 and 7.8 ± 2.3 on day 2 (P < 0.04). The mean number of action potentials fired by axotomized cells (n = 14) was 3.3 ± 0.5 on day 1 and 10.7 ± 2.0 on day 2 (P < 0.004). (There was no significant difference between the number of action potentials evoked on day 1 in the control and axotomized cells; P > 0.9, Mann-Whitney test.) Although both groups exhibited a significant reduction in spike accommodation on the second day of the experiment, the reduction was significantly greater in axotomized sensory neurons, just as in our first set of experiments. Thus whereas the mean increase in the number of action potentials was 3.8 ± 1.6 from day 1 to day 2 in the control group, the mean increase was 7.4 ± 2.1 in the axotomized group (P < 0.05, 1-tailed value, Mann-Whitney test).

In addition to reducing accommodation, axotomy had other long-term effects on the electrophysiological properties of isolated sensory neurons in culture. There was a significant decrease in the current required to evoke a single action potential in the cells following axotomy. The mean spike threshold for axotomized cells decreased from 0.53 nA on day 1 to 0.41 nA on day 2 (P < 0.004, n = 14). By contrast, the spike threshold did not change significantly in the control cells (0.50 nA on day 1 vs. 0.52 nA on day 2; P > 0.8, n = 14). In addition, axotomy produced an increase in the input resistance of the cell membrane of sensory neurons. The mean input resistance of the axotomized cells was 50.3 ± 3.8 MΩ on day 1 and 59.3 ± 4.2 MΩ on day 2 (P < 0.04, n = 14). The input resistance in the control cells, on the other hand, decreased from day 1 (61.3 ± 5.1 MΩ) to day 2 (50.1 ± 4.0 MΩ), although this decrease was not statistically significant (P > 0.09, n = 14). The difference between the mean input resistances of the control and axotomized groups on day 1 was not statistically significant (P > 0.1, Mann-Whitney test). Although axotomy significantly increased the input resistance of the sensory neurons, the 18% change in input resistance would seem unlikely to fully explain the more than threefold reduction in accommodation observed in axotomized neurons. Furthermore, Dale et al. (1987) found that repeated applications of 5-HT induced dramatic long-term reductions in spike accommodation in cultured sensory neurons—similar to those induced by axotomy—without causing a significant increase in input resistance. It is possible, however, that the relationship between input resistance and accommodation is highly nonlinear in sensory neurons.

We failed to find significant changes in other electrophysiological properties of sensory neurons after axotomy. Thus the duration of a single action potential increased for both axotomized cells (10.1 ± 2.4 ms on day 1 vs. 13.1 ± 3.7 ms on day 2,) and control cells (13.0 ± 3.6 ms on day 1 vs. 13.8 ± 5.4 ms on day 2; Fig. 2), but in neither group was this increase statistically significant (P > 0.5 for each comparison). Neither were there significant changes in the resting potentials of the sensory neurons in the control or axotomized groups. The mean resting potential of the control
FIG. 1. Axotomy causes hyperexcitability of isolated sensory neurons in culture. A: number of action potentials evoked in control (unaxotomized) neurons on days 1 and 2 in response to injections of different levels of depolarizing current. Graphs in A–C depict means ± SE. B: number of spikes evoked in neurons that were axotomized after electrophysiological testing on day 1. C: graph of the changes in number of spikes evoked on the 2 days of the experiment for axotomized and control cells. D: examples of the responses of a control cell to injections of positive current on days 1 and 2. Neuron fired 2 spikes in response to 1 nA of current on both days 1 and 2. It fired 7 spikes in response to 3 nA of current on day 1 and 12 spikes on day 2. E: examples of the responses of an axotomized cell to injections of positive current on days 1 and 2. Neuron fired 1 spike in response to 1 nA of current on day 1 and 7 spikes on day 2. It fired 8 spikes in response to 3 nA of current on day 1 and 31 spikes on day 2. Records for day 1 were made before axotomy. Scale bars = 40 mV and 500 ms.

cells (n = 14) was 44.6 ± 1.3 mV on day 1 and 43.0 ± 1.8 mV on day 2 (P > 0.4). The mean resting potential of the axotomized cells (n = 14) was 44.3 ± 1.3 mV on day 1 and 44.6 ± 1.2 mV on day 2 (P > 0.9).

In addition to the electrophysiological changes described above, we observed long-term structural changes in both control and axotomized sensory neurons in culture. In particular, the outgrowth of the neurons in both groups was more complex on the second day of the experiment, as reflected in the number of branch points on their neurites. Thus the mean number of branch points per cell for control sensory neurons (n = 14) went from 10 ± 1.7 on day 1 to 12.2 ± 2.2 on day 2 (P < 0.003; Fig. 3A). The mean number of branch points per cell for axotomized sensory neurons (n = 14) went from 9.1 ± 1.9 on day 1 to 13.6 ± 2.4 on day 2 (P < 0.001; Fig. 3B). As was the case for the reduction in accommodation, axotomy enhanced the increase in structural complexity observed on the second day of the experiments. This is reflected in a comparison of the difference scores for the changes in branch point number from day 1 to day 2 for the control and axotomized groups, which indicated that the number of branch points increased significantly more in the
axotomized sensory neurons than in the controls (P < 0.03, Mann-Whitney test; Fig. 3C). The difference between the mean number of branch points in the outgrowth of axotomized and control neurons on day 1 of the experiments was not significant (P > 0.6, Mann-Whitney test).

**Rp-cAMPS inhibits the increases in excitability and neuritic outgrowth due to axotomy**

It has been suggested that the previously reported long-term, injury-induced changes in *Aplysia* sensory neurons might be due to activation of PKA and its subsequent stimulation of cyclic AMP response-element-binding-protein (CREB)–driven gene expression (Walters and Ambron 1995). As a first step in determining the potential role of cAMP-inducible genes in the long-term alterations in sensory neurons in cell culture after axotomy, we tested whether an inhibitor of PKA, Rp-cAMPS, could block these long-term changes. The protocol for these experiments was similar to that of our earlier experiments with the exception that, after electrophysiological recording and morphological inspection, perfusion medium in the cell culture dish was replaced with culture medium containing Rp-cAMPS (500 μM) 2–3 min before any of the neurons in the dish underwent axotomy. Then, after performing the axotomies, the dish was placed back into the cell culture incubator. The cells remained in the Rp-cAMPS–containing culture medium for the entire 24-h period until electrophysiological recording on day 2 of the experiment.

The presence of Rp-cAMPS blocked long-term changes in accommodation in sensory neurons (Fig. 4A). Thus the mean number of action potentials evoked by 1 nA of current in control/Rp-cAMPS cells (n = 10) was 1.6 ± 0.2 spikes on day 1 and 1.5 ± 0.7 spikes on day 2 (P > 0.6), whereas the mean number of action potentials evoked in axotomized/Rp-cAMPS cells (n = 12) was 1.6 ± 0.2 spikes on day 1 and 1.6 ± 0.2 spikes on day 2 (P > 0.9). In addition, the change from day 1 to day 2 in the mean number of action potentials evoked by the 1-nA current pulse did not differ significantly between control/Rp-cAMPS cells and axotomized/Rp-cAMPS cells (P > 0.2, Mann-Whitney test; Fig. 4B). Rp-cAMPS also blocked the long-term structural changes in sensory neurons (Fig. 5, A and B). The mean number of branch points per cell for control/Rp-cAMPS neurons (n = 8) was 8.1 ± 2.0 on day 1 and 9.1 ± 1.6 on day 2 (P > 0.2). The mean number of branch points per cell for the axotomized/Rp-cAMPS neurons (n = 9) was 9.7 ± 1.7 on day 1 and 10.8 ± 1.5 on day 2 (P > 0.4). The change from day 1 to day 2 in the mean number of branch points per cell did not differ significantly between the control/Rp-cAMPS and axotomized/Rp-cAMPS neurons (P > 0.9, Mann-Whitney test; Fig. 5C). Finally, the presence of Rp-cAMPS blocked the changes in spike threshold and input resistance induced by axotomy. The mean level of current required to evoke a single action potential in the axotomized/Rp-cAMPS cells (n = 12) was 0.74 ± 0.05 nA on day 1 and 0.80 ± 0.07 nA on day 2 (P > 0.2). The mean input resistance of the axotomized/Rp-cAMPS cells (n = 12) was 44.6 ± 3.3 MΩ on day 1 and 41.0 ± 2.7 MΩ on day 2 (P > 0.3).

**Electrophysiological and structural alterations in control sensory neurons appear to be due to axotomy during cell culturing**

We were intrigued by the significant changes in both accommodation and neuritic outgrowth from day 1 to day 2 exhibited by the control sensory neurons (Figs. 1, A and D, and 3, A and C). Although these electrophysiological and structural changes were significantly less than those in the axotomized neurons, we wished to understand their origin. Injury-induced intracellular signals similar to those triggered by axotomy may have been stimulated by damage of control neurons due to their intracellular impalement on day 1. An alternative explanation, however, is suggested by the consideration that neurons in the control group had been axotomized during their dissociation from the CNS. Possibly, injury during dissociation stimulated the identical cellular pro-
Axotomy induces increased neurite outgrowth in cultured sensory neurons maintained in normal culture medium. A1: photomicrograph of a control sensory neuron on day 1. A2: photomicrograph of the control sensory neuron in A1 on day 2. Bar, 25 μm. B1: photomicrograph of an axotomized sensory neuron on day 1 before axotomy. →, point at which the cell’s neurite was severed. B2: photomicrograph of the sensory neuron in B1 immediately after axotomy. B3: photomicrograph of the sensory neuron in B1 and 2, on day 2. Note dramatically increased outgrowth compared with day 1. Bar, 25 μm. C: changes in the number of branch points for control and axotomized neurons maintained in normal culture medium throughout the experiment. *, difference between the two groups with respect to change in branch point number is statistically significant (see RESULTS).
Effects of axotomy on sensory neurons are mediated in part by intrinsic, cAMP-dependent cellular signals

The present results provide strong evidence that axonal injury per se can induce long-term electrophysiological and morphological changes in *Aplysia* sensory neurons similar to those previously observed after nerve crush in intact *Aplysia* or in the intact CNS of *Aplysia* (Clatworthy and Walters 1994; Gunstream et al. 1995; Steffensen et al. 1995; Walters et al. 1991). Specifically, we found that axotomizing isolated sensory neurons in cell culture produces a decrease in spike accommodation, a decrease in spike threshold, an increase in membrane resistance, and increased defasciculation and neuritic outgrowth. Thus these axotomy-induced effects do not depend on the presence of other, nonsensory neurons or glia, because such cells were absent from our cultures. The cell culture medium in our experiments consisted of 50% *Aplysia* hemolymph. As has been pointed out previously (e.g., Schacher and Proshansky 1983), hemolymph contains as-yet unidentified growth factors. Furthermore, the hemolymph also may have contained immunocytes because it was not filtered to remove immune cells. It is therefore possible that growth factors and/or immunocytes in the hemolymph contributed to at least some of axotomy’s effects on sensory neurons documented here (although see Ambron et al. 1996). We have yet to perform similar experiments in hemolymph-free medium to test this possibility. Nevertheless, we consider a significant contribution from immune cells to the effects we observed unlikely. Notice that even if growth factors or immunocytes were present in the hemolymph, their concentration in the cell culture medium would have been diluted by the L15, which constituted 50% of the cell culture medium (see Schacher and Proshansky 1983).

In addition to providing evidence that axonal damage generates intrinsic signals in sensory neurons that directly induce long-term, learning-like changes in these neurons, our results provide the first compelling evidence that injury activates the cAMP-dependent second messenger pathway within *Aplysia* sensory neurons. Axotomy’s electrophysiological and morphological effects on the sensory neurons are blocked when the axotomized sensory neurons are treated with the specific PKA inhibitor Rp-cAMPS. The ability of the PKA inhibitor to block injury’s long-term physiological and morphological changes in *Aplysia* sensory neurons, in turn, implicates PKA-dependent gene transcription in these long-term changes.

Many of axotomy’s effects on *Aplysia* sensory neurons are similar to those of 5-HT (see Introduction). The antiaccommodative effect of 5-HT on the sensory neurons is mediated by modulation of the S-type K⁺ current (IKS) (Baxter and Byrne 1990; Byrne and Kandel 1992; Ghirardi et al. 1992; Goldsmith and Abrams 1992; Klein et al. 1986); modulation of IKS in turn, appears to be predominately due to activation of PKA. However, 5-HT not only activates...
PKA within *Aplysia* sensory neurons but also activates protein kinase C (PKC) (Sacktor and Schwartz 1990; Sossin and Schwartz 1992). Recent evidence indicates that PKC plays an important role in the cellular changes underlying 5-HT–induced facilitation of sensorimotor synapses (Braha et al. 1993; Byrne and Kandel 1996; Ghirardi et al. 1992; Sugita et al. 1992, 1994). In particular, PKC-mediated changes in *Aplysia* sensory neurons are thought to mediate the so-called “spike-broadening independent” process induced by 5-HT, a process that may involve enhancement of transmitter mobilization or exocytosis (Byrne and Kandel 1996). In addition to modulating presynaptic mechanisms of transmitter release, 5-HT–induced activation of PKC also contributes to broadening of the sensory neuron’s action potential. This spike broadening appears to be due predominately to modulation of the slow, transiently activated K\(^+\) current (\(I_{K(V)}\)) (Baxter and Byrne 1989; Byrne and Kandel 1996). Interestingly, we did not find that axotomizing sensory neurons in vitro produced statistically significant spike broadening, at least in the case of single action potentials evoked by threshold levels of current. This contrasts with the results observed after nerve crush (Gunstream et al. 1995; Walters et al. 1991). The absence of a significant effect of axotomy on spike broadening in the case of cultured neurons may indicate that axotomy predominantly affects PKA levels in sensory neurons. However, we only examined spike duration at 24 h after in vitro axotomy, and spike broadening may develop relatively slowly compared with the increase in excitability after axotomy. Therefore, we cannot at present exclude the possibility that axotomy activates PKC, as well as PKA, within sensory neurons.

Our results imply that axotomy activates adenylate cyclase...
within the sensory neurons. How might injury trigger activation of adenylate cyclase? The most likely candidate signal by which axotomy activates adenylate cyclase is an elevation of intracellular Ca\(^{2+}\). Recently Ziv and Spira (1997) have shown that axotomy of isolated Aplysia buccal neurons in cell culture produces a large, transient rise in intracellular Ca\(^{2+}\) (to 300–500 \(\mu\)M) at the site of injury. By means of focal applications of the Ca\(^{2+}\) ionophore ionomycin, Ziv and Spira further showed that this localized elevation of intracellular Ca\(^{2+}\) was sufficient to trigger the morphological changes in the cultured neurons induced by axotomy. Specifically, focal application of ionomycin, like axotomy, triggered the formation of a growth cone from the cut end of the axon and the subsequent axonal defasciculation and neuritic outgrowth. The morphological remodeling of cultured Aplysia neurons observed by Ziv and Spira after axotomy appears identical to those we observed in the present study. This suggests that at least some of the long-term alterations in axotomized sensory neurons also were initiated by a large, transient rise in intracellular Ca\(^{2+}\). Because Aplysia neurons possess an adenylate cyclase, which is activated by Ca\(^{2+}\)/calmodulin (Abrams et al. 1991; Eliot et al. 1989), such a rise in intracellular Ca\(^{2+}\) would be expected to induce increased synthesis of cAMP within the neurons.

Intracellular injection of cAMP induces long-term structural changes in Aplysia sensory neurons similar to those we observed after axotomy (Nazif et al. 1991; Schacher et al. 1993). The cAMP-induced structural remodeling requires protein synthesis because it can be blocked by anisomycin, a protein synthesis inhibitor (see also Bailey et al. 1992b; O’Leary et al. 1995). These results suggest that a prolonged increase in intracellular cAMP induces protein synthesis in Aplysia sensory neurons and that this protein synthesis, in turn, causes long-term structural changes in the sensory neurons, including increased branching. Two recent findings provide additional support for such a scenario. First, Bailey et al. (1995) found that blocking the expression of Aplysia CCAAT enhancer-binding protein (ApCREB2)—an immediate early gene the transcription of which is induced by both cAMP and 5-HT and which is required for long-term facilitation of the sensorimotor synapses in Aplysia (Alberini et al. 1994)—also blocks the 5-HT–induced long-term structural changes in the sensory neurons. Second, Bartsch et al. (1995) found after injecting antiserum to Aplysia cell adhesion molecule (ApCAM) a constitutively expressed transcription factor that represses the cAMP-induced transcription required for long-term facilitation—into Aplysia sensory neurons, a single brief application of 5-HT (which normally induces only short-term facilitation) induces both long-term facilitation of sensorimotor synapses and long-term growth in sensory neurons. This work, together with our finding that Rp-cAMPS blocks the axotomy-induced increase in outgrowth and branching of cultured sensory neurons, suggests that axotomy leads to CREB1-driven transcription and translation in Aplysia sensory neurons (see Bartsch et al. 1995). Indeed, Alberini et al. (1994) have reported that dissection causes dramatic induction of ApC/EBP in the CNS of Aplysia.

One protein the synthesis of which may be altered by injury of sensory neurons is Aplysia cyclic AMP response element binding protein 2 (apCAM), a cell adhesion molecule in Aplysia (Barzilai et al. 1989; Mayford et al. 1992; Schacher et al. 1990). The synthesis of apCAM is decreased shortly after sensory neurons are exposed to 5-HT. In addition, preexisting apCAM molecules on the surface membrane of sensory neurons are rapidly down-regulated by 5-HT through endocytosis. [This endocytosis is itself dependent on protein synthesis (Bailey et al. 1992a).] Treating isolated sensory neurons in culture with monoclonal antibodies to apCAM for 24 h induces defasciculation and neuritic branching (Mayford et al. 1992) like that which we observed 24 h after axotomy (Fig. 3). In addition to possibly modulating the expression of genes associated with the down-regulation of apCAM, axonal injury also appears to alter the expression of genes for a variety of other proteins in Aplysia sensory neurons. Thus Noel et al. (1995) have found that, after crushing central and peripheral nerves, which contain the axons of the pleural sensory neurons, there is increased expression of mRNA for actin, intermediate filament protein, and calreticulin in pleural ganglia ipsilateral to the crushed nerves. Although these molecular changes were not specifically localized to the sensory neurons in Noel et al.’s study, it seems reasonable to assume that the changes occurred, to some extent, within sensory neurons, because sensory neurons constitute a significant proportion of the cells in the pleural ganglia (see Walters et al. 1983). The potential role of the molecular changes described by Noel et al. in the electrophysiological and morphological changes we observed remains to be determined.

We do not know the signaling pathway for the presumed genomic changes induced by axonal injury. However, the Rp-cAMPS results suggest that axotomy produces an elevation of cAMP within sensory neurons just as does repeated exposure of the sensory neurons to 5-HT. Furthermore, the large, prolonged increase in cytosolic cAMP induced by repeated 5-HT application has been shown to translocate the free catalytic subunit of PKA from the cytosol to the nucleus (Bacsak et al. 1993), where it then activates ApCREB1 (Bartsch et al. 1995; Kaang et al. 1993). Our results suggest that axotomy also may cause retrograde diffusion of the catalytic subunit of PKA to, and subsequent translocation into, the nucleus of the injured sensory neuron. One difficulty with this scheme, however, is that colchicine and nocodazole—drugs that disrupt active axonal transport—have been reported to block hyperexcitability of the sensory neurons after nerve crush in isolated preparations of the nervous system of Aplysia (Gunstream et al. 1995). These drugs would not be expected to interfere with simple diffusion of retrograde signaling molecules. However, the distance of the site of axonal injury from the cell soma in this study—10–20 mm—was significantly longer than those involved in our study, and axonal transport might be required to transport retrograde signals over such a distance.

Aplysia sensory neurons in dissociated cell culture are probably undergoing long-term, injury-induced cellular alterations

Since its development by Schacher and colleagues (Rayport and Schacher 1986; Schacher and Proshansky 1983), the Aplysia cell culture system has been a powerful tool for the analysis of cellular and molecular mechanisms of long-
term memory (e.g., Alberini et al. 1994; Bailey et al. 1992a; Bartsch et al. 1995; Dale et al. 1987, 1988; Dash et al. 1990; Glanzman et al. 1990; Lin and Glanzman 1994a,b; Mayford et al. 1992; Montarolo et al. 1986, 1988; Schacher et al. 1993). These studies have provided crucial insights into how learning-related stimuli induce persistent changes in neurons and synapses, insights that have proved to be of general importance for understanding learning and memory (see e.g., Bourchuladze et al. 1994; Huang et al. 1994; Yin et al. 1995; Yin et al. 1994). Our data introduce a potential complication in the interpretation of prior results from long-term studies of plasticity in Aplysia cell cultures. We found that control (unaxotomized) sensory neurons underwent some of the same electrophysiological and morphological changes as did axotomized sensory neurons, albeit to a lesser extent. Although we initially attributed these long-term changes in the control cells to injury-related signals induced by intracellular impalement, we discovered that unimpaled control sensory neurons exhibited the same amount of enhanced branching as did impaled control sensory neurons when inspected after 24 h. We also found that Rp-cAMPS suppressed the long-term structural alterations in both control and axotomized neurons (Fig. 5). This result indicates that injury-induced signals were elevated in the control neurons as well as in the axotomized neurons. It is likely that these injury-induced signals were due to the axotomy of the sensory neurons during cell culturing (see also Alberini et al. 1994). Our findings raise the possibility that injury-induced signals in sensory neurons might have been present in other long-term experiments involving cultured Aplysia neurons, a point that also was made by Gunstream et al. (1995) based on other data. The significance of this potential complication for previous observations from these experiments is unclear. Possibly, our results apply only to isolated sensory neurons in culture; contact with an appropriate target motor neuron may suppress the injury-related signals in the sensory neurons. Furthermore, most previous studies of long-term plasticity with cultured Aplysia synapses have used synapses that were 4–5 days old at the beginning of the studies, whereas our neurons had only been in culture for 2 days. We do not yet know whether isolated sensory neurons that have been in culture for 4–5 days, instead of just 2, would exhibit alterations in excitability and morphology similar to those we observed. Dale et al. (1987), however, reported that sensory neurons that had been isolated in culture for 4–5 days did not exhibit significant changes in spike accommodation during a 24 h period. (Dale et al. did not examine the sensory neurons for possible morphological alterations.)

An interesting question is why the effects of cell culturing-related axotomy of the sensory neurons appear to be less robust than the effects of in vitro axotomy. The answer to this question may lie in the results of Gunstream et al. (1995), who compared the effects of crushing the axons of pleural sensory neurons at distal and proximal sites in the nervous system of Aplysia. They found that proximal crush (at a distance of 10 mm away from the cell body) causes hyperexcitability to appear earlier than did distal crush (at a distance of 40 mm away from the cell body). Gunstream et al. argued that the relatively delayed onset of hyperexcitability after distal nerve crush reflects the additional time required for plasticity-signaling molecules to reach the soma from the distal crush site via retrograde axonal transport. Such an effect might explain why axotomized sensory neurons exhibited generally greater electrophysiological and morphological changes than did control neurons in our experiments. If so, one would predict that after several days in culture the electrophysiological and morphological characteristics of control sensory neurons would be indistinguishable from those of axotomized neurons.

On the resemblance between the long-term cellular changes induced by injury and those induced by learning

The similarity between the long-term changes in cultured sensory neurons we observed after axotomy and those associated with long-term sensitization in Aplysia is striking. The possible connection between the injury-induced and learning-related sets of long-term plastic changes is strengthened by our finding that many of the cellular changes induced by axotomy are blocked by the PKA inhibitor Rp-cAMPS. As discussed above, this result implicates CREB-driven expression in the long-term effects of axotomy. Recent work on vertebrate nervous systems also has provided parallels between gene expression stimulated by axonal injury and that which occurs during learning. Thus Thomas Herdegen and his colleagues have reported increased expression of a variety of transcription factors, including c-JUN, JUN D and KROX-24 (also termed Zif/268), in central and peripheral neurons after they have been axotomized or crushed (Fiallos-Estrada et al. 1993; Herdegen et al. 1992, 1993). Increased expression of these, as well as other, transcription factors in neurons has been associated with long-term, learning-related synaptic changes in vertebrates (Abraham et al. 1994; Cole et al. 1989; Demmer et al. 1993; Qian et al. 1993; Richardson et al. 1992; Wisden et al. 1990; Worley et al. 1993), particularly long-term potentiation and long-term depression. These close parallels between the cellular responses of neurons to injury and to learning-related activity should come as no surprise. Many of the processes that are initiated and controlled by the expression of transcriptional factors in axotomized neurons are likely to play important roles in the long-term cellular changes that underlie learning and memory (Walters and Ambron 1995). For example, increased expression of JUN and KROX-24 proteins have been related to sprouting by axons after injury (Herdegen et al. 1992). Axonal sprouting plays a critical role in long-term memory (Bailey and Chen 1988; Bailey and Kandel 1993; Glanzman et al. 1990; Nazif et al. 1991), and it is reasonable to suppose that the intracellular signals that trigger axonal sprouting in injury-related and learning-related plasticity will prove similar. An interesting difference between the in vitro morphological changes effected by axotomy and those effected by learning-related stimulation in Aplysia sensory neurons, however, is that axotomy produces significant outgrowth of isolated sensory neurons in culture, whereas repeated application of 5-HT does not alter the morphology of isolated sensory neurons; rather, the presence of the postsynaptic motor neuron is required for the 5-HT–induced morphological changes in presynaptic sensory neurons (Glanzman et al. 1990). [Notice that a postsynaptic signal is not similarly required for the long-term hyperexcitability of cultured sensory neurons induced by 5-HT (Dale et al. 1987).] There are two possible explanations for this apparent discrepancy. First, axonal injury may induce some intracellular signal, in
addition to elevated cAMP, required for enhanced neuritic outgrowth, which is not supplied by 5-HT in the absence of postsynaptic target. Such a signal might be activated by a postsynaptically derived Aplysia growth factor (see, e.g., Zhang et al. 1997) the release of which is stimulated by 5-HT. A second possible explanation is that 5-HT, but not axonal injury, also induces a signal that prevents enhanced neuritic outgrowth, and that this inhibition is removed by the presence of the postsynaptic cell. (For another example of a dissociation between the cellular effects of injury on Aplysia sensory neurons and those of 5-HT, see Povelones et al. 1997). As our knowledge about the nuclear and cytoplasmic signals activated in neurons by injury and those activated by memory-inducing stimuli increases, the challenge for the future will be to determine the subtle ways in which these two sets of signals differ.

We thank G. G. Murphy for assistance with the data analysis and figures. We also thank M. E. Barish, G. G. Murphy, M. Klein, and E. T. Walters for helpful comments on an earlier version of this paper.

This research was supported by National Institute of Neurological Disorders and Stroke Grant NS-29563, National Science Foundation Grant IBN-9410579, the Alzheimer’s Disease Program, State of California Grant 95-23335, and the Academic Senate of the University of California, Los Angeles to D. L. Glanzman.

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Received 19 November 1996; accepted in final form 11 November 1997.

REFERENCES


GHIRARDE, M., BRAHA, O., HOCHEMER, B., MONTAROLO, P. G., KANDEL, E. R.
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