Axonal Rejoining Inhibits Injury-Induced Long-Term Changes in *Aplysia* Sensory Neurons *In Vitro*

Supinder S. Bedi[1] **and David L. Glanzman**[1,2]

[1]*Department of Neurobiology, School of Medicine, University of California, Los Angeles, California 90095-1763, and*
[2]*Department of Physiological Science and the Brain Research Institute, University of California, Los Angeles, California 90095-1761*

Injury of *Aplysia* sensory neurons, both in the CNS and in dissociated cell culture, produces long-term changes in these cells, among which are hyperexcitability and enhanced neuritic outgrowth (hypermorphogenesis). These long-term, injury-induced changes are attributable, in part, to the generation of new intrinsic cellular signals. Little is known, however, about the signals that maintain homeostasis within sensory neurons. To elucidate the role of homeostatic signals in *Aplysia* sensory neurons, we investigated how axonal rejoining alters the cellular consequences of axotomy. Sensory neurons in dissociated cell culture were axotomized. In some cases, the distal segment of the severed axon was then removed; in other cases, the proximal and distal segments of the severed axon were permitted to rejoin. If the severed distal segment was left unmolested, then axonal rejoining invariably occurred within 7 hr. Surprisingly, we found that the characteristic long-term cellular consequences of axotomy were suppressed by axonal rejoining. The long-term axotomy-induced changes were not inhibited merely by contact between the severed axon and another, uninjured sensory neuron. These results indicate that long-term changes in sensory neurons induced by injury are attributable, in part, to prolonged disruption of a retrograde homeostatic signal that originates in the distal segment of the growing neurite and chronically suppresses hyperexcitability and hypermorphogenesis.

Key words: regeneration; homeostatic signals; neural plasticity; neural repair; axotomy; hyperexcitability; neuritogenesis

Injury induces long-term electrophysiological and morphological changes in sensory neurons of the marine snail *Aplysia californica*. Among these changes are hyperexcitability and enhanced neuritic outgrowth (Walters et al., 1991; Gunstream et al., 1995; Steffensen et al., 1995; Bedi et al., 1998). These long-term cellular changes resemble those that occur during long-term learning in *Aplysia*, particularly during long-term sensitization (Bailey and Chen, 1983; Scholz and Byrne, 1987; Bailey and Chen, 1988; Scholz and Byrne, 1988; Glanzman et al., 1990; Nazif et al., 1991; O'Leary et al., 1995). This resemblance has led to the suggestion that the cellular processes activated by neuronal injury and those activated during learning converge (Walters and Ambron, 1995).

Injury-induced changes in sensory neurons of *Aplysia* appear to be attributable, at least in part, to intrinsic signals, because they are observed after neuritotomy (hereafter axotomy) of isolated sensory neurons in dissociated cell cultures that lack both nonsensory neurons and glia (Ambron et al., 1996; Bedi et al., 1998). Among the intrinsic signals that appear to be required for the injury-induced electrophysiological and morphological changes are PKA and PKC (Bedi et al., 1998; Liao et al., 1999). In addition to intrinsic signals being activated by injury within sensory neurons, the long-term effects of injury may require the interruption of continuous signals that maintain homeostasis within the neu-

rons (Wu et al., 1993). A previous report by Gunstream et al. (1995) concluded that interruption of continuous homeostatic signals does not underlie injury-induced hyperexcitability of sensory neurons, because application of drugs that disrupt axonal transport to uninjured axons of sensory neurons in an *in vitro* preparation did not induce hyperexcitability. These experiments, however, did not rule out the possibility that the homeostatic signals are transported to the soma of sensory neurons by retrograde diffusion. Furthermore, it is possible that interruption of some homeostatic signal, although necessary, is insufficient to trigger long-term changes in sensory neurons and that a second injury-induced signal is also required. Finally, Gunstream et al. (1995) did not look at potential long-term morphological alterations in sensory neurons after disruption of axonal transport.

We have tested the potential involvement of disruption of homeostatic signals in long-term, injury-induced changes in *Aplysia* sensory neurons using isolated neurons in dissociated cell culture. In the present experiments, the major neurite of cultured sensory neurons was severed, and the effects of this injury on the excitability and morphology of neurons were examined 24 hr later. In our previous experiments (Bedi et al., 1998), the distal segment of the severed neurite was removed. In some of the present experiments, however, the distal segment was not removed after axotomy but rather was left in place. In such instances, we observed that the proximal and distal segments of the severed neurite invariably rejoin. Furthermore, this rejoining suppressed the long-term hyperexcitability and hypermorphogenesis otherwise induced by axotomy. Some of our results have been published previously in abstract form (Bedi and Glanzman, 1997, 2000).

MATERIALS AND METHODS

Cell cultures. All experiments were performed on isolated sensory neurons in dissociated cell culture. The cell cultures consisted of mech-

anosensory neurons removed from ventrocaudal clusters of pleural ganglia of *Aplysia californica* (Walters et al., 1983). The cell culture methods have been described previously (Schacher and Proshansky, 1983; Bedi et al., 1998). The cultures contained neither nonsensory neurons nor glia. The sensory neurons were individually dissociated and placed into cell culture sufficiently far apart from one another that their processes did not touch, except for those experiments (Axotomy-Contact) in which the effects of neuronal contact subsequent to axotomy were assessed (see below). The cultures were kept at 18–22°C for 2 d before the start of the experiments. All cultures were 2 d old at the start of the experiments.

The data were obtained from a total of 31 cell-culture dishes. Nine culture dishes were used for the comparison between the axotomy (*n* = 12 cells) and control-A (*n* = 11 cells) groups (see Results), nine dishes were used for the comparison between the rejoining (*n* = 12 cells) and control-R (*n* = 13 cells) groups, and nine dishes were used in experiments (Axotomy-Contact) in which we tested whether mere contact between a severed axon and the neurite of another, intact neuron could suppress long-term injury-induced changes. Finally, four dishes were used for experiments in which a rejoining neuron was filled with fluorescent dye to confirm cytoplasmic continuity of the rejoined axon. There were two types of Axotomy-Contact experiments: those in which the severed axon contacted the neurite of another cell within 7 hr (Axotomy-Contact$_{EARLY}$; *n* = 5) and those in which the severed axon contacted the neurite of another cell within 24 hr (Axotomy-Contact$_{LATE}$; *n* = 9). The axotomy and rejoining experiments used approximately equal numbers of experimental and control cells per dish.

Electrophysiology. The experiments were performed at room temperature (20–22°C). Before the start of each recording session, the hemolymph-containing culture medium (Schacher and Proshansky, 1983) was washed out of the culture dish and replaced with perfusion medium [50% sterile artificial seawater (ASW) and 50% sterile Liebowitz-15 (L-15); Sigma, St. Louis, MO] plus appropriate salts. The 50% ASW/50% L-15 medium was perfused through the culture dish at 0.4 ml/min. Sensory neurons were impaled with sharp microelectrodes (15–20 MΩ). Standard techniques were used for intracellular stimulation and recording (Lin and Glanzman, 1994; Bedi et al., 1998). After impaling a neuron, we first measured its membrane potential, input resistance, and spike threshold. We then tested its excitability. This was done by injecting a 2 sec pulse of 2 nA of positive current into the neuron. After the electrophysiological tests had been performed, some sensory neurons (axotomized and rejoining) were axotomized with a glass microneedle. The major neurite of the neurons was severed approximately halfway down its length. For the axotomy group, the severed distal portion of the neurite was removed from the culture dish. For the rejoining group, the distal segment of the severed neurite was not removed; instead, the proximal stump of the neurite was permitted to rejoin with the distal segment. In the Axotomy-Contact experiments, other neurons were cultured with the neuron to be axotomized before axotomy, or other, freshly dissociated neurons were placed beside an axotomized neuron immediately after axotomy. After the experimental manipulations were completed on day 1 of the experiment, the cell cultures were replaced into hemolymph-containing cell culture medium. After ~24 hr, the electrophysiological properties and structure (see below) of the neurons were reassessed.

Assessment of morphology. We photographed the entire outgrowth of each sensory neuron on day 1 and day 2 of each experiment with a CCD camera (Hamamatsu Photonics, Oak Brook, IL) or digital camera (Polaroid DMC 1; Polaroid, Penfield, NY). The complexity of the structure of the neuron was assessed by counting the number of branch points on its neurites (Bedi et al., 1998). If a neuron had only a single main neurite with no branch points, the neuron was assigned a branch point value of 1.0.

Lucifer yellow (Molecular Probes, Eugene, OR) was used in some rejoining experiments to confirm the cellular continuity of the rejoined proximal and distal segments of the severed axon. For dye filling, the tip of a microelectrode was filled with Lucifer yellow (10 mM) and the electrode was then backfilled with the recording electrode solution (Lin and Glanzman, 1994). Hyperpolarizing current (0.5 nA, 30 min) was applied to the dye-containing electrode to inject the dye into a neuron.

Statistics. Within-group statistical comparisons (day 1 vs day 2) were made with paired *t* tests. Between-group comparisons were made with Mann–Whitney *U* tests. Potential differences among the four experimental groups were assessed by one-way ANOVA. When the variances of the four groups were not homogeneous, as indicated by a Bartlett's test, we used a nonparametric ANOVA (Kruskal–Wallis test). All reported levels of statistical significance represent two-tailed values unless otherwise indicated.
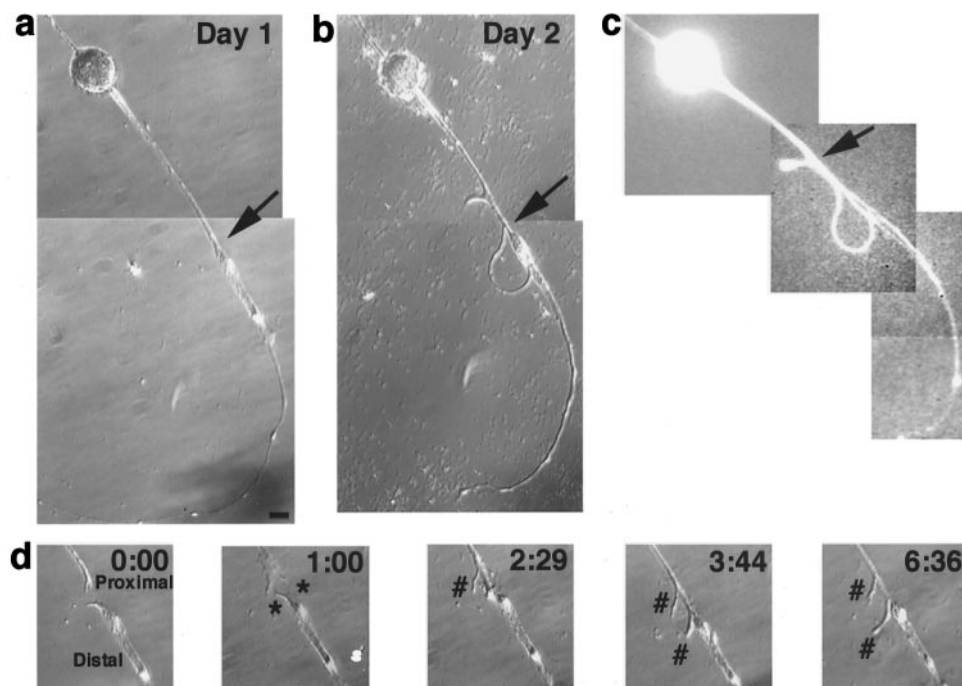
RESULTS

There were four separate sets of cell cultures. In one set, the major neurite of some neurons was completely severed (axotomized group) on day 1 of the experiment, whereas other neurons in the same dishes were left intact (control-A group). In a second set of cultures, some neurons were axotomized and then the proximal and distal segments were permitted to rejoin (rejoining group). Other neurons in the same dishes as the rejoining neurons were left intact (control-R group). The effects of either axotomy alone or axotomy followed by rejoining could therefore be determined through direct statistical comparisons between neurons that received one of these two experimental treatments and control neurons in the same dish. There were no statistically significant differences between the control-A and control-R groups on any of our measurements (below). In the third set of cell cultures, pairs of sensory neurons were placed near one another during culturing (Axotomy-Contact$_{LATE}$ group). One of the pairs of sensory neurons was axotomized and then the severed axon of the neuron was allowed to contact the nonaxotomized neuron of the pair. The purpose of including the Axotomy-Contact experiments in this study was to determine whether mere contact with another neuron after axotomy inhibited the long-term effects of axotomy. In the Axotomy-Contact$_{LATE}$ experiments, we did not constrain the time to contact between the severed axon and the other neuron of the pair; rather, we allowed 24 hr for this neuronal contact to occur. In a fourth set of experiments (Axotomy-Contact$_{EARLY}$), however, the time required for a severed axon to contact another neuron was kept to within 7 hr, the time normally required for rejoining of the proximal and distal segments of the axotomized neurites in the rejoining group.

Neurites of sensory neurons can rejoin after axotomy

The severed distal segments of axons (anucleate axons) in the nervous system of invertebrates and of some lower vertebrates can survive for weeks to years (Krasne and Lee, 1977a,b; Bittner, 1991). Isolated distal segments of severed axons have also been reported to survive for days in certain mouse strains (Perry et al., 1990; Brown et al., 1994). We found that the severed distal segment of the major neurite of sensory neurons in culture did not degenerate but survived for hours. Furthermore, if permitted, the proximal and distal segments of the axotomized sensory neurons invariably rejoined (Fig. 1*a,b*). This axonal rejoining took 2–7 hr to occur and was mediated by outgrowth from the cut ends of both the proximal and distal segments (Fig. 1*d*). In some experiments (*n* = 4), the rejoining sensory neuron was filled with Lucifer yellow on day 2 to confirm that there was cytoplasmic continuity between the rejoined proximal and distal segments (Fig. 1*c*). [Note that these dye-injected neurons, like the other rejoining neurons described below, did not exhibit either hyperexcitability (*p* > 0.8) or enhanced neuritic outgrowth (*p* > 0.6) on day 2.]

Axonal rejoining inhibits long-term hyperexcitability of axotomized sensory neurons in cell culture

Aplysia sensory neurons accommodate to prolonged pulses of positive current (Klein et al., 1986; Baxter and Byrne, 1989). Injury disrupts this accommodation, both *in vivo* (Walters et al., 1991) and *in vitro* (Gunstream et al., 1995; Ambron et al., 1996; Bedi et al., 1998). Therefore, we tested the effect of rejoining on

Figure 1. Axotomized sensory neurons in culture will rejoin with severed distal segments of their axons. Time-lapse photomicrographs showing axonal rejoining in culture. *a,* A sensory neuron *in vitro* before axotomy on day 1. *b,* Same sensory neuron on day 2 after axotomy and rejoining. *c,* Same neuron on day 2 after filling with Lucifer yellow. The dye indicates that rejoining re-established cytoplasmic continuity between the distal and proximal segments of the axon. *Arrows* in *a–c* indicate the site of axotomy and rejoining. *d,* Time-lapse views of rejoining of distal and proximal segments of an axotomized neuron. The neuron is the same as in *a–c.* Note growth of filopodia (*asterisks*) from the severed end of the distal axonal segment 1 hr after axotomy and the subsequent retraction of the filopodia after rejoining (at 3 hr and 44 min after axotomy). Also visible are new filopodia growing from the tip of the proximal segment (*pound signs*), some of which do not retract after rejoining has occurred. Rejoining is mediated by outgrowth from the distal segment as well as from the proximal segment. Scale bar, 10 μm.

the injury-induced hyperexcitability of sensory neurons. In agreement with previous results (Ambron et al., 1996; Bedi et al., 1998), we found that sensory neurons were significantly more excitable 24 hr after axotomy. Axotomized cells ($n = 12$) fired significantly more action potentials in response to a 2 sec injection of positive current (2 nA) on day 2 of the experiments than on day 1 (7.6 $\pm$ 1.8 vs 3.2 $\pm$ 0.7 spikes; $t = 2.58$; $p < 0.03$; Fig. $2a_1,a_2$). Control cells in the same dishes as the axotomized cells (control-A cells, $n = 10$) did not exhibit significantly greater excitability on day 2 than on day 1 (4.6 $\pm$ 1.3 vs 3.3 $\pm$ 0.7 spikes; $p > 0.1$; Fig. $2b_1,b_2$). Furthermore, sensory neurons whose neurites were severed and then permitted to rejoin, as in Figure 1 (rejoining cells, $n = 12$), did not fire significantly more action potentials in response to injected current on day 2 than on day 1 (5.6 $\pm$ 1.6 vs 3.7 $\pm$ 0.7 spikes; $p > 0.3$; Fig. $3a_1,a_2$). Neither was the excitability of control cells in the same dishes as the rejoining cells (control-R cells, $n = 11$) significantly greater on day 2 than day 1 (4.7 $\pm$ 1.4 vs 2.6 $\pm$ 0.5 spikes; $p > 0.2$; Fig. $3b_1,b_2$). An ANOVA indicated that there were no significant differences among the four experimental groups with respect to the number of action potentials evoked on day 1 ($p > 0.6$).
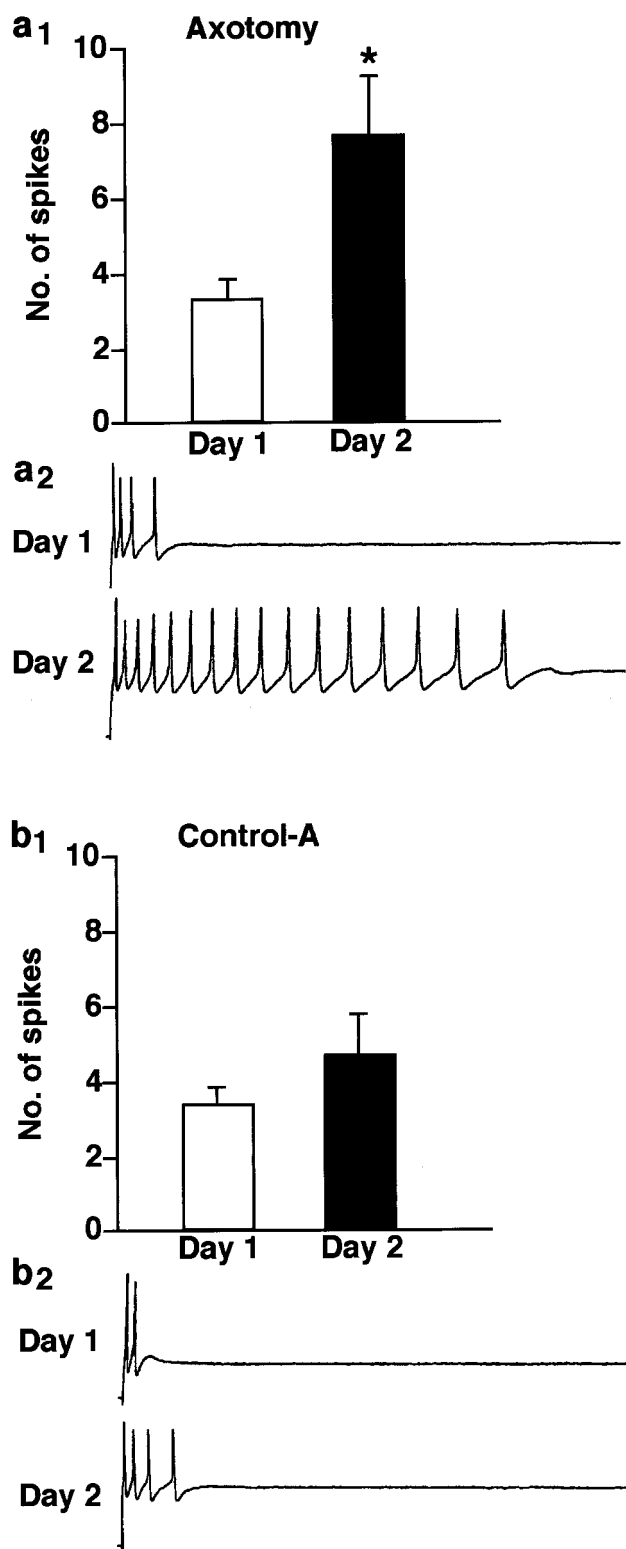
There was no significant difference between the mean resting potential of the cells in the axotomy and control-A groups on day 1 (47.3 $\pm$ 1.3 vs 46.8 $\pm$ 1.2 mV). Furthermore, the mean resting potential of the cells did not change significantly from day 1 to day 2 in either the axotomy ($p > 0.5$) or control-A ($p > 0.9$) groups. Neither was there a significant difference between the mean resting potential of the cells in the rejoining and control-R groups on day 1 (46.7 $\pm$ 2.2 vs 45.2 $\pm$ 1.5 mV). The mean resting potential of the control-R cells did not change significantly from day 1 to day 2 ($p > 0.5$). However, we did observe a small but statistically significant decrease in the resting potential of cells in the rejoining group (46.7 $\pm$ 2.2 mV on day 1 and 42.8 $\pm$ 1.3 mV on day 2; $t = 2.30$; $p < 0.05$). We have no explanation for this decrease and did not observe any other abnormalities in the rejoining neurons.

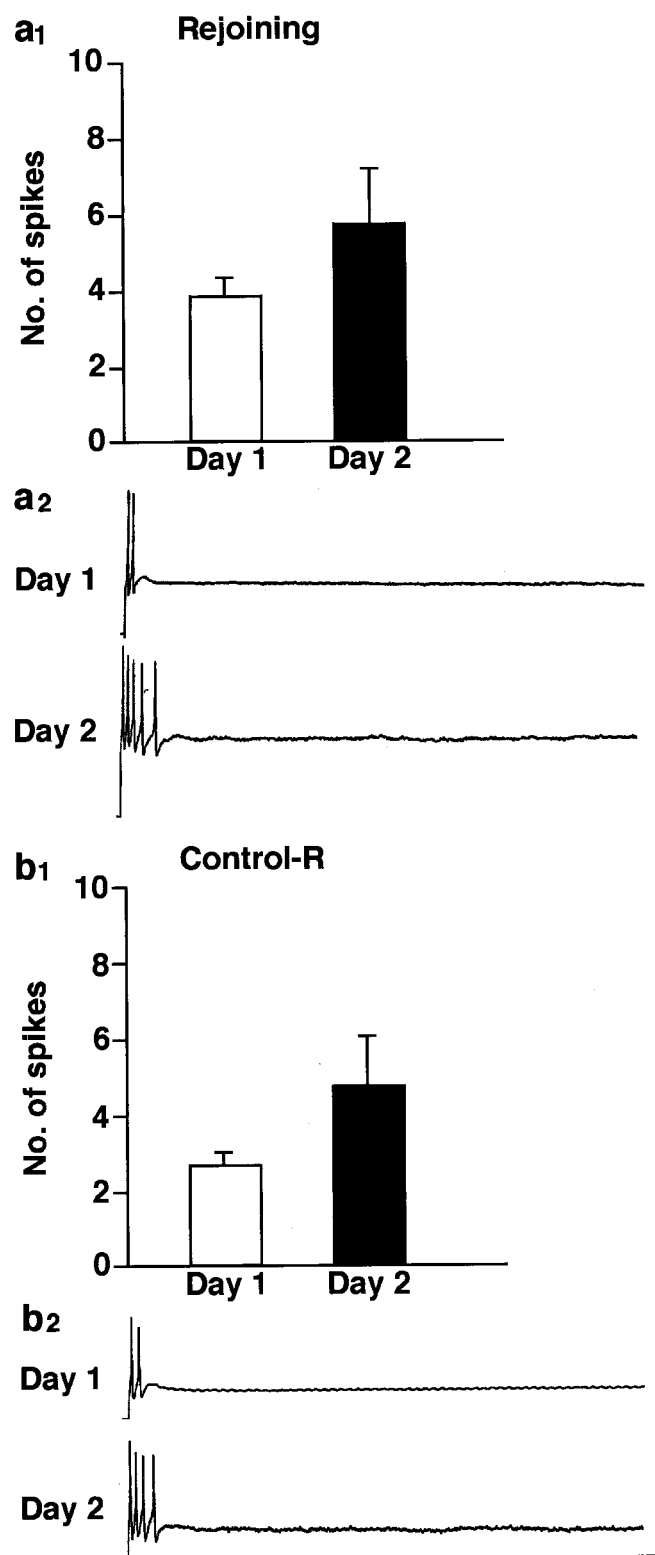Notice that, although not statistically significant, the excitability

of control-A and control-R cells consistently increased over the 24 hr of the experiments (Figs. $2b_1,b_2$ and $3b_1,b_2$). A long-term increase in the excitability of control sensory neurons was also observed in our previous *in vitro* study [Bedi et al. (1998), their Fig. 1]. We attribute this modest increase in the excitability of the control neurons to injury-induced signals resulting from axotomy of the sensory neurons during dissociation and culturing.

Axonal rejoining inhibits the enhanced hypermorphogenesis induced by axotomy in sensory neurons in cell culture

The morphology of sensory neurons, as reflected in the number of neuritic branch points, increased significantly in all groups between days 1 and 2. In agreement with our previous results (Bedi et al., 1998), the increase in neuritic branch points was significantly greater in axotomized cells than in control cells. The mean number of branch points per cell for control-A cells ($n = 11$) was 14.5 $\pm$ 2.9 on day 1 and 19.0 $\pm$ 3.6 on day 2 ($t = 3.45$; $p < 0.007$; Fig. $4a_1,a_2$). The mean number of branch points per cell for axotomized cells ($n = 12$) was 8.6 $\pm$ 2.1 on day 1 and 21.6 $\pm$ 4.7 on day 2 ($t = 3.64$; $p < 0.004$; Fig. $4b_1,b_2$). The change in the number of branch points was significantly greater for the axotomized cells than for the control-A cells. The difference in the number of branch points per cell between days 1 and 2 was 13 $\pm$ 3.6 for the axotomized group and 4.6 $\pm$ 1.3 for the control-A group (Mann–Whitney U test; $U = 34.5$; $p = 0.05$; Fig. $4c$). We did not observe a significant difference between the rejoining and control-R groups with respect to the morphological changes from day 1 to day 2. The number of branch points on the neurites of control-R cells ($n = 13$) went from 10.0 $\pm$ 1.89 on day 1 to 13.8 $\pm$ 2.5 on day 2 ($t = 2.68$; $p < 0.02$; Fig. $5a_1,a_2$). The mean number of branch points on the neurites of rejoining cells ($n = 12$) went from 7.7 $\pm$ 1.7 on day 1 to 12.0 $\pm$ 1.2 on day 2 ($t = 3.61$; $p < 0.005$; Fig. $5b_1,b_2$). However, the change in the mean number of branch points per cell from day 1 to day 2 did not differ significantly between the control-R and rejoining groups (3.8 $\pm$ 1.4 vs 4.3 $\pm$ 1.2; $p > 0.6$; Fig. $5c$). The differences among the four experimen-
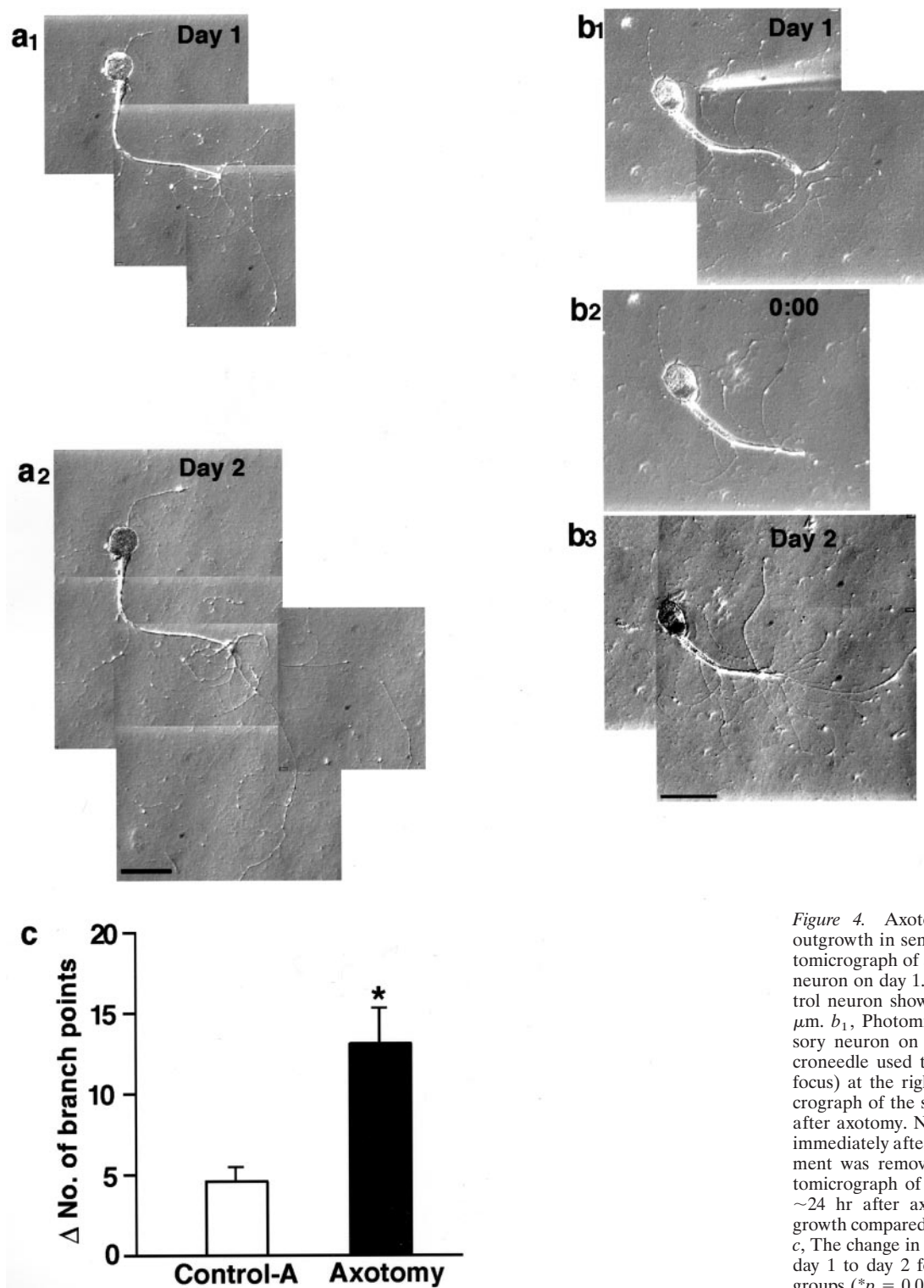
Figure 2. Axotomy induces long-term hyperexcitability of isolated sensory neurons in culture. a_1, Number (mean ± SEM) of spikes evoked in axotomized neurons on days 1 and 2 in response to injections of positive current (*$p < 0.03$). a_2, Examples of the responses of an axotomized neuron to current injections on days 1 and 2. The neuron fired 4 spikes on day 1 and 16 spikes on day 2. b_1, Number (mean ± SEM) of spikes evoked in control (unaxotomized) neurons on days 1 and 2 in response to injections of positive current. b_2, Examples of the responses of an unaxotomized neuron to current injections on days 1 and 2. The neuron fired two spikes on day 1 and four spikes on day 2. Calibration, 20 mV, 50 msec.

Figure 3. Axonal rejoining inhibits the long-term hyperexcitability of axotomized sensory neurons in culture. a_1, Number (mean ± SEM) of spikes evoked in rejoining neurons on days 1 and 2 in response to injections of positive current. a_2, Examples of the responses of a rejoining neuron to current injections on days 1 and 2. The neuron fired two spikes on day 1 and five spikes on day 2. b_1, Number (mean ± SEM) of spikes evoked in control (unaxotomized) neurons on days 1 and 2 in response to injections of positive current. b_2, Examples of the responses of an unaxotomized neuron to current injections. The neuron fired two spikes on day 1 and four spikes on day 2. Calibration, 20 mV, 50 msec.

Figure 4. Axotomy induces enhanced neuritic outgrowth in sensory neurons in culture. a_1, Photomicrograph of a control (unaxotomized) sensory neuron on day 1. a_2, Photomicrograph of the control neuron shown in a_1 on day 2. Scale bar, 100 μm. b_1, Photomicrograph of an axotomized sensory neuron on day 1 before axotomy. The microneedle used to cut the axon is visible (out of focus) at the right of the cell body. b_2, Photomicrograph of the sensory neuron in b_1 immediately after axotomy. Note that *0:00* represents the time immediately after axotomy. The severed distal segment was removed in this experiment. b_3, Photomicrograph of the sensory neuron in b_1 and b_2 ~24 hr after axotomy. Note the increased outgrowth compared with that in b_1. Scale bar, 100 μm. c, The change in the number of branch points from day 1 to day 2 for the control-A and axotomized groups (*$p = 0.05$). Graph depicts means ± SEM.

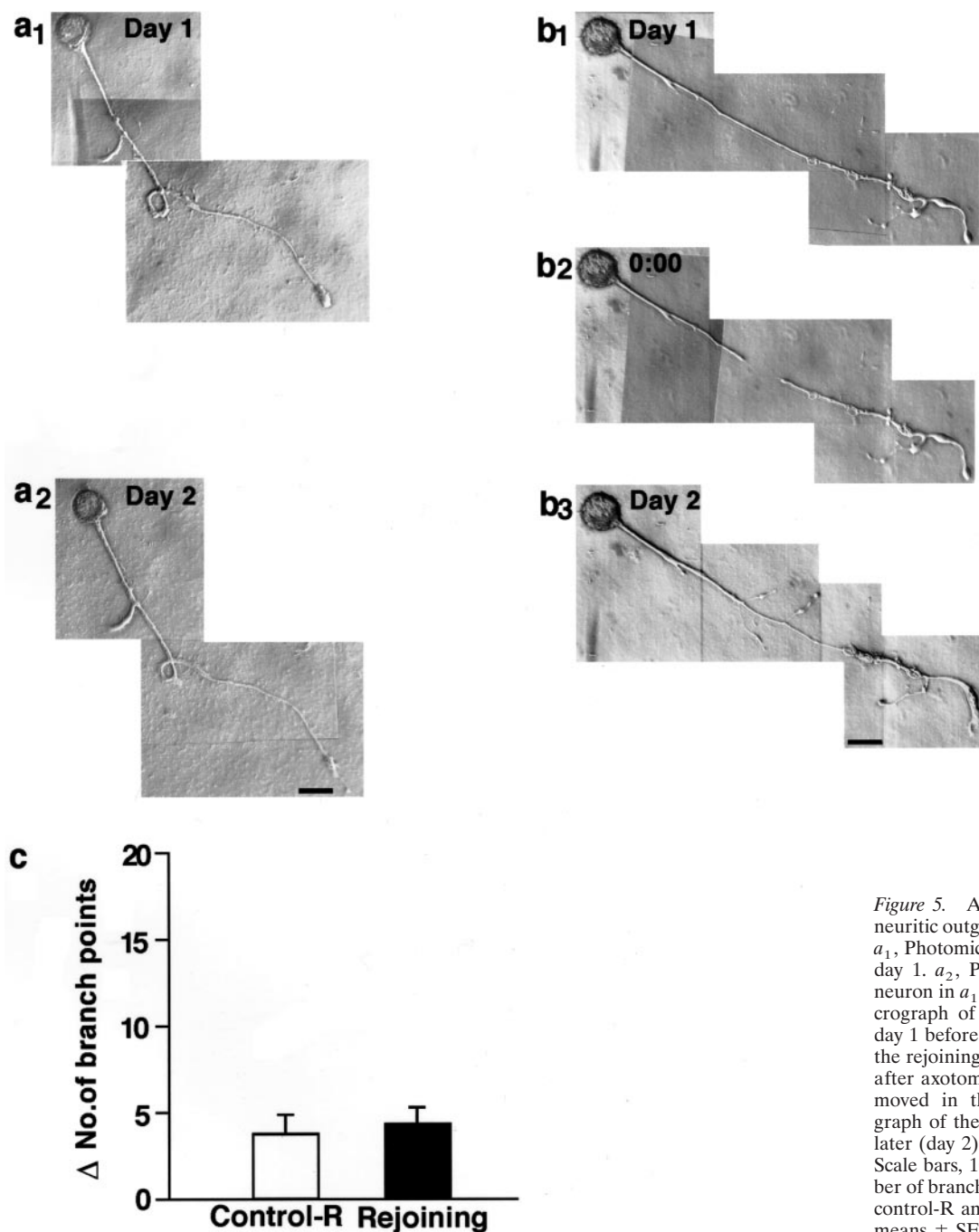tal groups with respect to the number of neuritic branches per cell on day 1 were not significant (ANOVA; $p > 0.1$).

Contact with another sensory neuron after axotomy does not inhibit hyperexcitability

An alternative explanation for the effect of axonal rejoining on excitability is that inhibition of the long-term changes is mediated not by rejoining per se but rather simply by contact between the proximal segment of the axotomized cell and the surviving distal segment, which is no longer recognized as belonging to the same cell. We tested this alternative hypothesis (i.e., that mere neuronal contact can suppress the axotomy-induced, long-term cellular changes) using hyperexcitability as a measure of these changes.

We conducted two separate sets of experiments in which axotomized sensory neurons were permitted to contact other sensory neurons in dissociated cell culture. In the first set (Axotomy-
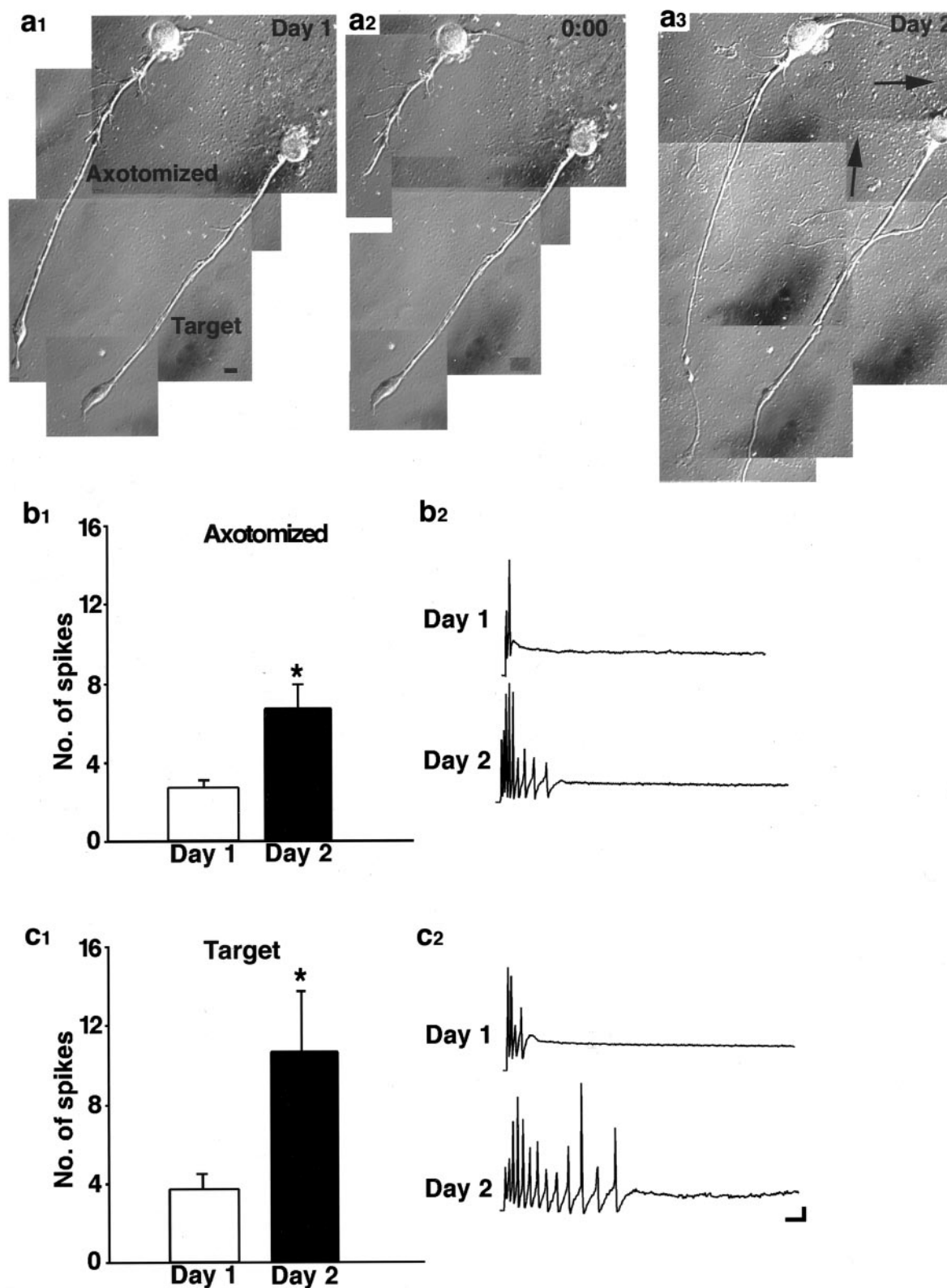
Figure 5. Axonal rejoining inhibits increased neuritic outgrowth in cultured sensory neurons. a_1, Photomicrograph of a control-R neuron on day 1. a_2, Photomicrograph of the control-R neuron in a_1 ~24 hr later (day 2). b_1, Photomicrograph of a sensory neuron (rejoining) on day 1 before axotomy. b_2, Photomicrograph of the rejoining sensory neuron in b_1 immediately after axotomy. The distal segment was not removed in this experiment. b_3, Photomicrograph of the rejoining sensory neuron ~24 hr later (day 2). Note that the axon has rejoined. Scale bars, 100 μm. *c*, The change in the number of branch points from day 1 to day 2 for the control-R and rejoining groups. Graph depicts means ± SEM.
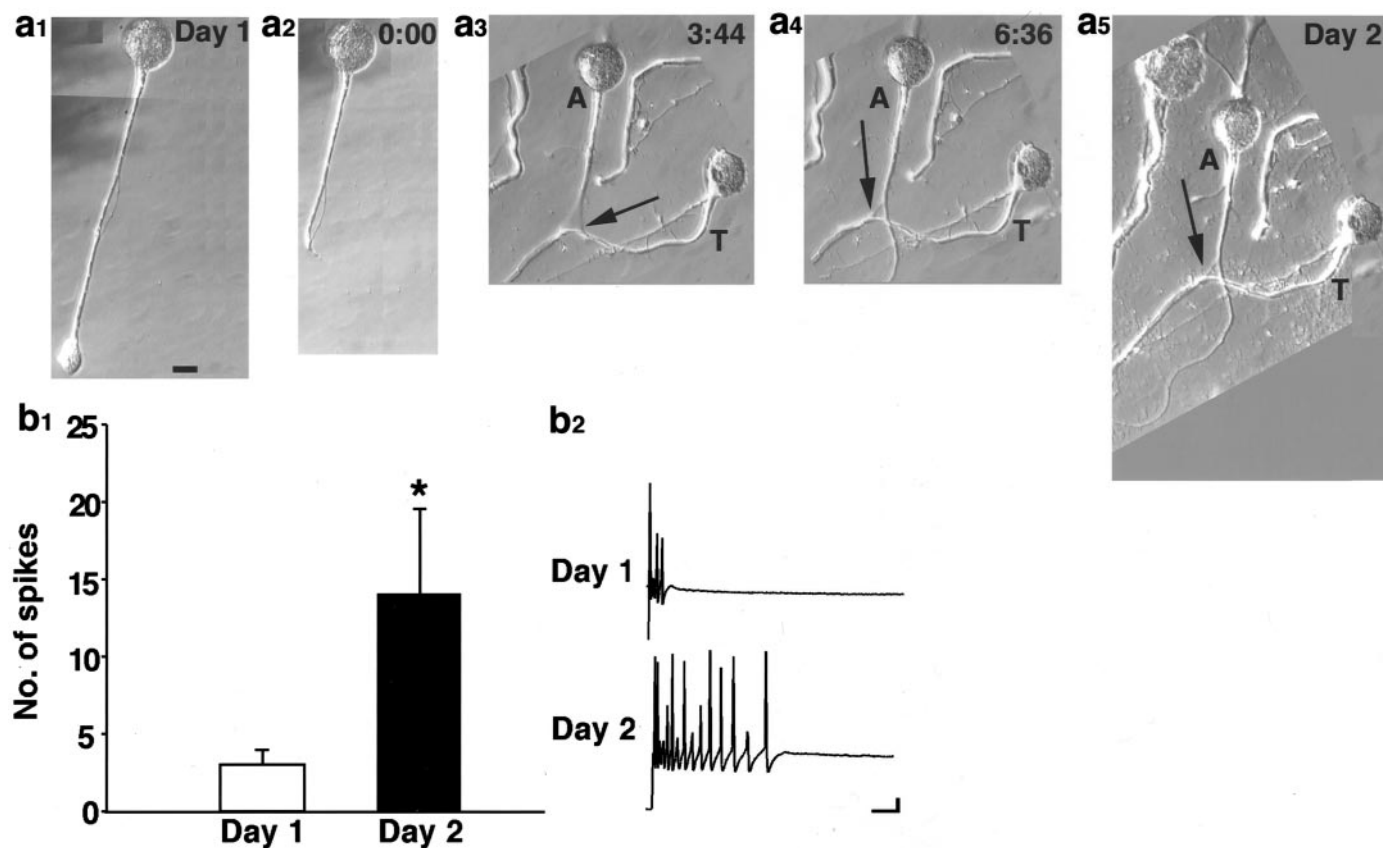
Contact$_{LATE}$), pairs of sensory neurons were placed together during culturing. The two sensory neurons of a pair were positioned so that their processes did not touch each other (Fig. $6a_1$). On the second day after culturing, each pair of neurons was inspected under a microscope to ensure that the processes of the two neurons were not in physical contact. (If the two neurons were in contact, the pair was discarded.) In these experiments, we tested the excitability of both neurons in the pair. After its excitability had been tested, one neuron of each pair was axotomized and its distal segment was removed. Then the culture dish was placed back in the incubator. After 24 hr, the pair was visually inspected to determine whether the proximal segment of the axotomized sensory neuron had grown out and contacted the unaxotomized (target) neuron of the pair. Contact between the

axotomized and target neurons occurred in ~30% of the cases (Fig. $6a_2,a_3$). If contact did not occur, the pair was discarded. If contact did occur, the excitability of the axotomized and target neurons was retested. We found that subsequent contact between the axotomized and target neurons did not inhibit the development of long-term hyperexcitability in the axotomized neuron. Axotomized neurons ($n = 9$) fired significantly more action potentials in response to an injection of positive current on day 2 of the experiments than on day 1 (6.8 ± 1.2 vs 2.8 ± 0.4 spikes; $t = 3.9$; $p < 0.005$; Fig. $6b$). Surprisingly, we also found a significant increase in excitability in the uninjured target neuron ($n = 9$) [10.7 ± 3.1 spikes (day 2) vs 3.5 ± 0.8 spikes (day 1); $t = 2.8$; $p < 0.03$; Fig. $6c$].

The finding of a spread of long-term hyperexcitability from the

Figure 6. Results of Axotomy-Contact$_{LATE}$ experiments. Contact between an axotomized sensory neuron and a target sensory neuron over 24 hr does not inhibit long-term hyperexcitability in the axotomized neuron and causes transfer of hyperexcitability to the target neuron. a_1, Photomicrographs of a neuron to be axotomized (*Axotomized*) and a neuron that it will later contact after axotomy (*Target*). The neurons are shown on day 1 before the axotomy. Scale bar, 10 μm. a_2, Neurons shown in a_1 immediately after the neuron at the *upper left* has been axotomized. a_3, The two neurons on day 2. *Arrows* indicate points of contact between the axotomized and target neurons. b_1, Number (mean $\pm$ SEM) of spikes evoked in axotomized neurons on days 1 and 2 in response to injections of positive current (*$p < 0.005$). b_2, Examples of responses of an axotomized neuron to current injections on days 1 and 2. The neuron fired two spikes on day 1 and nine spikes on day 2. c_1, Number (mean $\pm$ SEM) of spikes evoked by target neurons on days 1 and 2 in response to injections of positive current (*$p < 0.03$). c_2, Examples of the responses of a target neuron to current injections on days 1 and 2. The neuron fired 4 spikes on day 1 and 13 spikes on day 2. Calibration, 10 mV, 125 msec.

Figure 7. Results of Axotomy-Contact$_{EARLY}$ experiments. Axonal contact between the axotomized sensory neuron and a target neuron <7 hr after axotomy does not inhibit long-term hyperexcitability in the axotomized neuron. a_1, Photomicrograph of a neuron on day 1 before axotomy (Axotomy-Contact$_{EARLY}$ experiment). Scale bar, 10 μm. a_2, Same neuron as in a_1 immediately after axotomy. a_3, Axotomized neuron (A) of a_1 and a_2 initially contacting a target neuron (T). Time of initial contact is 3 hr and 44 min after axotomy. a_4, Same view as that of a_3 at 6 hr and 36 min after axotomy. a_5, Same view at day 2. *Arrows* indicate the point of contact between the axotomized and target neurons. b_1, Number (mean ± SEM) of spikes evoked in axotomized neurons on days 1 and 2 in response to injections of positive current (*p < 0.05, one-tailed t test). b_2, Examples of the responses of an axotomized neuron to current injections on days 1 and 2. The neuron fired 5 spikes on day 1 and 16 spikes on day 2. Calibration, 10 mV, 125 msec.

injured neuron to the uninjured target neuron suggests that neuronal contact per se does not inhibit axotomy-induced hyperexcitability. In the Axotomy-Contact$_{LATE}$ experiments, however, we did not attempt to limit the time to contact between the pairs of neurons to the time (<7 hr) required for axonal rejoining. Consequently, these experiments did not exclude the possibility that, to suppress the axotomy-induced changes, neuronal contact had to occur within a critical time period. Therefore, we performed another set of experiments (Axotomy-Contact$_{EARLY}$) in which the time for neuronal contact between the axotomized and target neurons was limited to <7 hr. This proved to be extremely difficult. Two sensory neurons could not be placed too close together during culturing, because they would almost invariably contact each other before we could begin the axotomy experiment (Glanzman et al., 1991). However, if the sensory neurons were placed sufficiently far apart during culturing that contact did not occur during the first 2 d in culture (Fig. 6a_1), then the axotomized neuron typically required >7 hr to contact the target neuron. We found two protocols that enabled contact between an axotomized neuron and a target neuron within 7 hr but prevented contact between the to-be-axotomized neuron and another neuron before actual axotomy. In the first protocol, the sensory neuron to be axotomized was cultured alone. Next, immediately after axotomy, three or four freshly dissociated sensory neurons were placed into the culture dish near the axotomized neuron

(Fig. 7a_3). Each of these neurons served as a potential target neuron. In a small number of cases ($n = 3$), the neurite from the axotomized neuron succeeded in contacting one of the potential target neurons within 7 hr. Typically, the axotomized neuron did not contact at least one of the other neurons within 7 hr, usually because the neurites of the freshly dissociated neurons did not stick to the bottom of the culture dish soon enough. In the second protocol we used to obtain neuronal contact within 7 hr, a potential target was placed next to the neuron to be axotomized 12 hr before the start of an experiment. (Thus, the to-be-axotomized and target neurons were placed into culture 36 hr apart.) In two cases, we obtained neuronal contact between an axotomized and a target neuron within 7 hr using this second protocol. Therefore, our results for the Axotomy-Contact$_{EARLY}$ group are based on five successful experiments. The time required to make contact in these five experiments was 4–5 hr after axotomy. We found that mere neuronal contact between the extending proximal segment of the axotomized neuron and a neurite of the target neuron within this period did not prevent axotomy-induced hyperexcitability. The excitability of the axotomized neurons increased significantly from day 1 to day 2 (3.2 ± 0.8 vs 14 ± 5.5 spikes; $t = 2.24$; $p < 0.05$; one-tailed t test; Fig. 7b). (We did not measure the excitability of the target neurons in the Axotomy-Contact$_{EARLY}$ experiments.) Therefore, we conclude that the suppression of the axotomy-induced long-term hyperex-

citability that we observed in the rejoining group was indeed attributable to axonal rejoining and not merely to contact between the proximal segment of the severed axon and another neuronal entity.
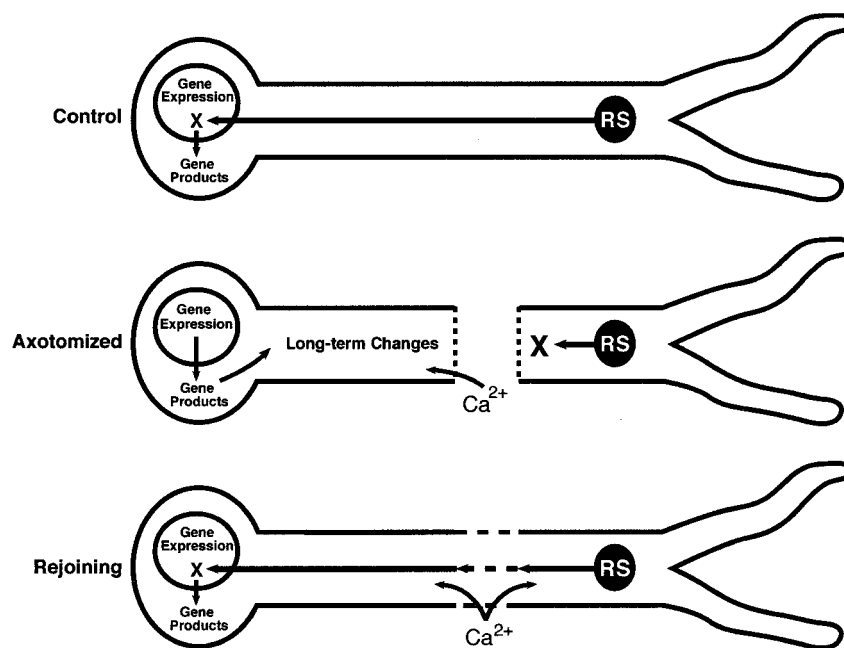
DISCUSSION

We have shown that if the severed distal segment is left undisturbed after axotomy of the major neurite of cultured *Aplysia* sensory neurons, the distal segment will survive and the proximal segment will rejoin with the surviving distal segment, thereby establishing cytoplasmic continuity between the two segments (Fig. 1). The rejoining of the distal and proximal ends of the severed neurite inhibits long-term electrophysiological and morphological changes that otherwise would be induced by axotomy (Ambron et al., 1996; Bedi et al., 1998). This inhibition of the axotomy-induced long-term changes does not appear to be attributable simply to contact between the distal and proximal segments, because contact between the proximal segment of an axotomized sensory neuron and the neurites of another target neuron did not inhibit the long-term hyperexcitability of the axotomized neuron, even when such contact occurred within the same time window as for axonal rejoining (Axotomy-Contact$_{EARLY}$ experiments; Fig. 7). Another argument against the idea that neuronal contact, rather than actual axonal rejoining, suppresses the long-term cellular changes normally induced by axonal injury is that we observed an apparent spread of hyperexcitability from the axotomized neurons to the uninjured target neurons in the Axotomy-Contact$_{LATE}$ experiments (Fig. 6). This result suggests that axotomy causes some hyperexcitability-inducing signal to be generated in the axotomized neuron and that this signal can be transferred to another uninjured neuron via neuritic contact. The most probable mechanism for such transfer is electrical junctions between the axotomized and target neurons, although we did not test for such electrical coupling in the present experiments. It has been demonstrated previously that axotomy promotes the formation of electrical and dye coupling between neurons of the snail *Helisoma* (Murphy et al., 1983). We do not know the identity of the hyperexcitability-inducing signal transferred from an axotomized neuron to a target neuron. However, we will attempt to determine the identity of this signal in future experiments. It will also be interesting to determine whether a similar transfer of long-term hyperexcitability from injured to uninjured sensory neurons can occur in the intact CNS of *Aplysia*.

Spira and colleagues (Benbassat and Spira, 1993; Spira et al., 1993) have shown previously that the distal segments of axotomized nonsensory interneurons of *Aplysia* in dissociated cell culture can survive and rejoin with the proximal segment of the main neurite. Moreover, they reported that the isolated distal segments can remain alive and capable of extending new neurites for 2–14 d *in vitro*. We have extended these findings to isolated sensory neurons of *Aplysia* in culture. An important question is whether the survival of the isolated distal segment of axotomized *Aplysia* sensory neurons and the rejoining of the distal and proximal segments depend on *de novo* protein synthesis. Benbassat and Spira (1993) originally concluded that survival and neuritic extension by anucleate neuritic segments of nonsensory neurons *in vitro* is attributable to utilization of pre-existing proteins and not to *de novo* protein synthesis. However, more recent evidence indicates that isolated neuritic segments of nonsensory neurons are capable of protein synthesis for up to 5 d after axotomy (Oren et al., 1997). In addition, isolated axons of *Lymnaea* are capable of synthesizing proteins when foreign mRNA is injected into axons lacking their somatas (Van Minnen et al., 1997). Furthermore, Martin et al. (1997) have shown that anucleate processes of *Aplysia* sensory neurons in culture are also able to synthesize proteins. In our experiments, the cell cultures contained only isolated sensory neurons; there were no glia or nonsensory neurons in the cultures. Moreover, the sensory neurons in the rejoining experiments were placed too far from each other to permit proteins to pass from an intact neuron to a severed neurite. Consequently, the survival of the severed distal segment cannot be attributed to donated proteins. In addition, neuritic rejoining was mediated by neuritic extension from the distal segment as well as from the proximal segment (Fig. 1*d*). These facts support the idea that both neuritic outgrowth from the distal segment and neuritic rejoining in our experiments were mediated by protein synthesis. Preliminary data suggest that the rejoining requires protein synthesis. We have found that anisomycin, a protein synthesis inhibitor, disrupts axonal rejoining (Bedi and Glanzman, 2000).

The present results confirm previous reports (Ambron et al., 1996; Bedi et al., 1998) that axotomy of isolated sensory neurons in dissociated cell culture reproduces many of the long-term cellular changes induced by injury of sensory neurons in the CNS of *Aplysia* (Walters et al., 1991; Gunstream et al., 1995; Steffensen et al., 1995). Among these long-term changes are hyperexcitability and hypermorphogenesis. The results from previous *in vitro* experiments indicate that at least some of the signals induced by injury are probably intrinsic to the sensory neurons. However, the present results indicate that injury-induced, intrinsic signals by themselves are insufficient to trigger hyperexcitability and hypermorphogenesis in sensory neurons. Rather, neuritic damage must be accompanied by disruption of one or more retrograde homeostatic signals. Furthermore, the disruption of the homeostatic signals must be relatively prolonged, because axonal rejoining, which occurs 2–7 hr after axotomy, suppresses the long-term cellular changes. We hypothesize that this suppression is attributable to the reinstatement of one or more homeostatic signals originating from the distal segment of the main sensory neurite (Fig. 8).

The present results qualify the conclusions of a previous study of injury-induced long-term changes in *Aplysia* sensory neurons. Gunstream et al. (1995) concluded that long-term hyperexcitability of axonally damaged sensory neurons did not depend on disruption of a continuous homeostatic signal, because application of blockers of axonal transport to undamaged sensory neurons in isolated central ganglia of *Aplysia* did not induce hyperexcitability. Our experiments were performed on cultured sensory neurons. It is possible that culturing sensory neurons somehow alters their ability to regulate their excitability, making them more susceptible to the effects of interruption of homeostatic signals. It is also possible that some regulative factor, perhaps originating in glia or nonsensory neurons, renders sensory neurons in the intact CNS relatively refractory to the interruption of retrograde homeostatic signals. Yet another explanation for the apparent discrepancy between the present results and those of Gunstream et al. (1995) is that the critical homeostatic signal is transported to the soma of sensory neurons by retrograde diffusion rather than by retrograde axonal transport. [Interestingly, positive injury-induced signals (those that are triggered in the axons by injury and whose arrival at the soma stimulates long-term hyperexcitability of sensory neurons) do seem to move by retrograde transport, because inhibitors of axonal transport can block injury-induced hyperexcitability (Gunstream et al., 1995).]

Figure 8. Model for the effect of axotomy and axonal rejoining on sensory neurons. According to the model, an inhibitory signal (RS) originates in the distal end of the sensory neuron and is continually transported or diffuses retrogradely to the cell nucleus. There it blocks gene expression, as indicated by *X*. In intact neurons, the RS maintains homeostatic levels of excitability and morphological outgrowth. Axotomy disrupts the RS and also produces a sharp rise in $[Ca^{2+}]_i$. These two cellular events result in long-term changes, possibly involving gene expression, among which are hyperexcitability and enhanced neuritic outgrowth (via defasciculation and sprouting) (Mayford et al., 1992). The reinstatement of the RS via axonal rejoining suppresses the long-term electrophysiological and morphological changes that would otherwise be caused by axotomy, despite the local rise in $[Ca^{2+}]_i$ in the transected axon. Note that the RS may represent multiple molecular signals.

Finally, as suggested above, the apparent discrepancy between the present results and those of Gunstream et al. (1995) may indicate that the disruption of a retrograde homeostatic signal, by itself, is insufficient to trigger long-term hyperexcitability in sensory neurons; rather, some positive injury-induced signal is also required (Ambron et al., 1995). Our evidence that disruption of retrograde, suppressive signal contributes to long-term changes in neurons is supported by a study by Smith and Skene (1997) of adult DRG neurons. This study found that blocking axonal transport in intact DRG neurons before transferring the neurons into cell culture resulted in a pattern of elongating *in vitro* outgrowth of the DRG neurons that closely resembled the *in vitro* outgrowth of DRG neurons cultured after axotomy.

An interesting question is how hyperexcitability is transferred from an axotomized neuron to a target neuron, as we observed in our Axotomy-Contact$_{LATE}$ experiments (Fig. 6). We presume that this transfer occurs despite the presence of retrograde, suppressive signals in the intact target neuron. It is possible that axotomy-induced positive signals can induce hyperexcitability despite the presence of retrograde, suppressive signals, given a sufficiently prolonged and/or massive exposure to the positive signals. According to this idea, the reason why reinstatement of the retrograde, suppressive signals is sufficient to inhibit hyperexcitability (as well as hypermorphogenesis) in the case of the rejoining neurons is that rapid axonal rejoining within 2–7 hr somehow inhibits or disrupts the positive signals stimulated by axotomy.

In summary, a necessary condition for the induction of axotomy-induced long-term electrical and morphological changes in sensory neurons of *Aplysia* appears to be the interruption of a continuous retrograde signal (RS) that normally acts to suppress these changes (Fig. 8). We do not yet know for how long RS must be interrupted before the long-term changes are irreversibly triggered in axotomized neurons. But the critical period for the disruption of RS must be on the order of hours, because neuritic rejoining requires 2–7 hr (Fig. 1). A possible candidate for RS is a tyrosine kinase activated by the binding of a growth factor to receptors on the distal tip of the axon of the sensory neuron

(Riccio et al., 1997). Our cell-culture medium consisted of 50% *Aplysia* hemolymph, which contains unidentified growth factors (Schacher and Proshansky, 1983). Ambron et al. (1996) reported that axotomy can cause long-term hyperexcitability of sensory neurons in cell culture in the absence of hemolymph. We have found that, although long-term hyperexcitability of axotomized sensory neurons in culture does not require the presence of hemolymph in the cell-culture solution (S. S. Bedi and D. L. Glanzman, unpublished observations), the enhanced neuritogenesis observed in axotomized sensory neurons (Fig. 4) does require hemolymph (Bedi and Glanzman, unpublished observations). This result suggests that the hemolymph may be the source of positive, growth-inducing signals as well as inhibitory signals. Furthermore, taken together with the results of Ambron et al. (1996), our present finding that axonal rejoining suppresses hyperexcitability as well as hypermorphogenesis may indicate that there are multiple RSs. The RS chronically suppresses neuritogenesis.

The present results extend the parallel between the long-term changes induced in *Aplysia* sensory neurons during long-term behavioral sensitization and those induced by axonal injury (Walters et al., 1991; Walters and Ambron, 1995; Bedi et al., 1998). As has been noted previously, long-term memory depends on the removal of inhibitory constraints on cellular changes such as axonal outgrowth (Abel et al., 1998). For example, repeated applications of serotonin, the transmitter that mediates sensitization in *Aplysia* (Glanzman et al., 1989), removes the repression of *Aplysia* cAMP response element-binding protein 1 (ApCREB1) by ApCREB2 in sensory neurons, thereby permitting the defasciculation and outgrowth of sensory neurites (Bartsch et al., 1995). Similarly, our results point to the presence of a retrograde, inhibitory signal in sensory neurons that normally suppresses hyperexcitability and neuritic outgrowth and is removed by axotomy. It seems increasingly likely that many of the cellular signals that mediate the response of *Aplysia* sensory neurons to learning-related stimuli also mediate their response to axonal injury.

A major conclusion from our results is that long-term neuronal changes are not an inevitable consequence of axotomy. Reinstate-

ment of homeostatic signals by axonal rejoining can suppress hyperexcitability and enhanced neuritogenesis in sensory neurons even hours after axonal injury. This finding may have potential clinical implications.

REFERENCES

Abel T, Martin KC, Bartsch D, Kandel ER (1998) Memory suppressor genes: inhibitory constraints on the storage of long-term memory. Science 279:338–341.

Ambron RT, Dulin MF, Zhang XP, Schmied R, Walters ET (1995) Axoplasm enriched in a protein mobilized by nerve injury induces memory-like alterations in *Aplysia* neurons. J Neurosci 15:3440–3446.

Ambron RT, Zhang XP, Gunstream JD, Povelones M, Walters ET (1996) Intrinsic injury signals enhance growth, survival, and excitability of *Aplysia* neurons. J Neurosci 16:7469–7477.

Bailey CH, Chen M (1983) Morphological basis of long-term habituation and sensitization in *Aplysia*. Science 220:91–93.

Bailey CH, Chen M (1988) Long-term memory in *Aplysia* modulates the total number of varicosities of single identified sensory neurons. Proc Natl Acad Sci USA 85:2373–2377.

Bartsch D, Ghirardi M, Skehel PA, Karl KA, Herder SP, Chen M, Bailey CH, Kandel ER (1995) *Aplysia* CREB2 represses long-term facilitation: relief of repression converts transient facilitation into long-term functional and structural change. Cell 83:979–992.

Baxter DA, Byrne JH (1989) Serotonergic modulation of two potassium currents in pleural sensory neurons of *Aplysia*. J Neurophysiol 62:665–679.

Bedi SS, Glanzman DL (1997) Axotomy-induced long-term electrophysiological and morphological changes in isolated *Aplysia* sensory neurons are inhibited by axonal rejoining. Soc Neurosci Abstr 23:1959.

Bedi SS, Glanzman DL (2000) Inhibition of protein synthesis disrupts axonal rejoining following axotomy in *Aplysia* sensory neurons in cell culture. Soc Neurosci Abstr 26:1525.

Bedi SS, Salim A, Chen S, Glanzman DL (1998) 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.

Benbassat D, Spira ME (1993) Survival of isolated axonal segments in culture: morphological, ultrastructural, and physiological analysis. Exp Neurol 122:295–310.

Bittner GD (1991) Long-term survival of anucleate neurons and its implications for nerve regeneration. Trends Neurosci 14:188–193.

Brown MC, Perry VH, Hunt SP, Lapper SR (1994) Further studies on motor and sensory nerve regeneration in mice with delayed Wallerian degeneration. Eur J Neurosci 6:420–428.

Glanzman DL, Mackey SL, Hawkins RD, Dyke AM, Lloyd PE, Kandel ER (1989) Depletion of serotonin in the nervous system of *Aplysia* reduces the behavioral enhancement of gill withdrawal as well as the heterosynaptic facilitation produced by tail shock. J Neurosci 9:4200–4213.

Glanzman DL, Kandel ER, Schacher S (1990) Target-dependent structural changes accompanying long-term synaptic facilitation in *Aplysia* neurons. Science 249:799–802.

Glanzman DL, Kandel ER, Schacher S (1991) Target-dependent morphological segregation of *Aplysia* sensory outgrowth in vitro. Neuron 7:903–913.

Gunstream JD, Castro GA, Walters ET (1995) Retrograde transport of plasticity signals in *Aplysia* sensory neurons following axonal injury. J Neurosci 15:439–448.

Klein M, Hochner B, Kandel ER (1986) Facilitatory transmitters and cAMP can modulate accommodation as well as transmitter release in *Aplysia* sensory neurons: evidence for parallel processing in a single cell. Proc Natl Acad Sci USA 83:7994–7998.

Krasne F, Lee SH (1977a) Regenerating afferents establish synapses with a target neuron that lacks its cell body. Science 198:517–519.

Krasne F, Lee SH (1977b) Survival of functional synapses on crustacean neurons lacking cell bodies. Brain Res 121:43–57.

Liao X, Gunstream JD, Lewin MR, Ambron RT, Walters ET (1999) Activation of protein kinase A contributes to the expression but not the induction of long-term hyperexcitability caused by axotomy of *Aplysia* sensory neurons. J Neurosci 19:1247–1256.

Lin XY, Glanzman DL (1994) Long-term potentiation of *Aplysia* sensorimotor synapses in cell culture: regulation by postsynaptic voltage. Proc R Soc Lond B Biol Sci 255:113–118.

Martin KC, Casadio A, Zhu HEY, Rose JC, Chen M, Bailey CH, Kandel ER (1997) Synapse-specific, long-term facilitation of *Aplysia* sensory to motor synapses: a function for local protein synthesis in memory storage. Cell 91:927–938.

Mayford M, Barzilai A, Keller F, Schacher S, Kandel ER (1992) Modulation of an NCAM-related adhesion molecule with long-term synaptic plasticity in *Aplysia*. Science 256:638–644.

Murphy AD, Hadley RD, Kater SB (1983) Axotomy-induced parallel increases in electrical and dye coupling between identified neurons of *Helisoma*. J Neurosci 3:1422–1429.

Nazif FA, Byrne JH, Cleary LJ (1991) cAMP induces long-term morphological changes in sensory neurons of *Aplysia*. Brain Res 539:324–327.

O'Leary FA, Byrne JH, Cleary LG (1995) Long-term structural remodeling in *Aplysia* sensory neurons requires de novo protein synthesis during a critical time period. J Neurosci 15:3519–3525.

Oren R, Dormann A, Benbassat D, Spira ME (1997) Long term survival of isolated axonal segments as revealed by in vitro studies. In: Neurochemistry: cellular, molecular, and clinical aspects (Teelken T, Korf J, eds), pp 647–653. New York: Plenum.

Perry VH, Brown MC, Lunn ER (1990) Very slow retrograde and Wallerian degeneration in the CNS of C57B/Ola mice. Eur J Neurosci 3:102–105.

Riccio A, Pierchala BA, Ciarallo CL, Ginty DD (1997) An NGF-TrkA-mediated retrograde signal to transcription factor CREB in sympathetic neurons. Science 277:1097–1100.

Schacher S, Proshansky E (1983) Neurite regeneration by *Aplysia* neurons in dissociated cell culture: modulation by *Aplysia* hemolymph and the presence of the initial segment. J Neurosci 3:2403–2413.

Scholz KP, Byrne JH (1987) Long-term sensitization in *Aplysia*: biophysical correlates in tail sensory neurons. Science 235:685–687.

Scholz KP, Byrne JH (1988) Intracellular injection of cAMP induces a long-term reduction of neuronal K^+ currents. Science 240:1664–1666.

Smith DS, Skene JH (1997) A transcription-dependent switch controls competence of adult neurons for distinct modes of axon growth. J Neurosci 17:646–658.

Spira ME, Benbassat D, Dormann A (1993) Resealing of the proximal and distal cut ends of transected axons: electrophysiological and ultrastructural analysis. J Neurobiol 24:300–316.

Steffensen I, Dulin MF, Walters ET, Morris CE (1995) Peripheral regeneration and central sprouting of sensory neuron axons in *Aplysia californica* following nerve injury. J Exp Biol 198:2067–2078.

Van Minnen J, Bergman J, Van Kesteren E, Smit A, Geraerts W, Lukowiak K, Hasan S, Syed N (1997) *De novo* protein synthesis in isolated axons of identified neurons. Neuroscience 80:1–7.

Walters ET, Ambron RT (1995) Long-term alterations induced by injury and by 5-HT in *Aplysia* sensory neurons: convergent pathways and common signals? Trends Neurosci 18:137–142.

Walters ET, Byrne JH, Carew TJ, Kandel ER (1983) Mechanoafferent neurons innervating tail of *Aplysia*. I. Response properties and synaptic connections. J Neurophysiol 50:1522–1542.

Walters ET, Alizadeh H, Castro GA (1991) Similar neuronal alterations induced by axonal injury and learning in *Aplysia*. Science 253:797–799.

Wu W, Mathew TC, Miller FD (1993) Evidence that the loss of homeostatic signals induces regeneration-associated alterations in neuronal gene expression. Dev Biol 158:456–466.