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Similar Neuronal Alterations Induced by Axonal Injury and Learning in *Aplysia*

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Learning in the marine mollusk *Aplysia* has been associated with enhanced sensory function, expressed in mechanosensory neurons as (i) decreases in action potential threshold, accommodation, and afterhyperpolarization, and (ii) increases in action potential duration, afterdischarge, and synaptic transmission. These alterations also occur, with a delay, after sensory axons are injured under conditions in which synaptic transmission is severely reduced. The latency and specificity of injury-induced alterations indicate that induction signals are generated at the site of injury and conveyed centrally by axonal transport. Similarities in neuronal modifications support the hypothesis that some memory mechanisms evolved from mechanisms of injury-induced sensory compensation and repair.

BEHAVIORAL SENSITIZATION AND classical conditioning in various species have been correlated with a persistent increase in the excitability of selected neurons, often expressed as a decrease in action potential threshold or an increase in firing rate during depolarizing test stimuli (*1, 2*). Increased excitability is also seen in diverse neurons after injury of their peripheral axons (*3*). These observations, and the presumed ability of hyperexcitability to compensate for loss of sensory function in an area of injury, led to the suggestion that some learning mechanisms evolved from long-lasting adaptive responses to cellular injury in primitive sensory neurons (*4*).

We have begun to test this hypothesis by comparing injury-induced and learning-induced plasticity in the same neurons: nociceptive mechanosensory neurons in the mollusk *Aplysia*. The most dramatic plasticity reported for these cells occurs when the animal receives noxious stimulation within the receptive field of the neuron during aversive training, and this plasticity is mediated in part by activity-dependent effects of neuromodulators released on or near the sensory neuron soma (*2, 5*). However, because noxious stimuli can damage peripheral axons, we wondered whether additional signals for long-term plasticity in these cells are generated at the sites of axonal injury.

Experiments were performed on sensory neurons that have their somata in the ventrocaudal (VC) clusters of the pleural ganglia (Fig. 1A). The axons of these neurons project exclusively through the adjacent pedal ganglion and ipsilateral pedal nerves to innervate the ipsilateral body surface (*6*). Mechanosensory neurons in *Aplysia* usually show marked action potential accommodation during prolonged depolarization (*7*); action potentials (spikes) can only be initiated at the beginning of a depolarizing pulse (Fig. 1B, control), even if large depolarizing currents are applied. To investigate the effects of axon injury on accommodation, we anesthetized each animal and, through a small incision, crushed all of the pedal nerves coming from one pedal ganglion (*8*). After 1 to 23 days the pedal-pleural ganglia were dissected, and sensory neurons in each VC cluster examined.

We tested action potential accommodation by injecting a 1-s depolarizing pulse into the soma (Fig. 1B) (*9*). Sensory clusters subjected to axon crush 4 days earlier responded with significantly more action potentials (exhibiting less accommodation and thus greater excitability) than contralateral control clusters with uncrushed axons by a paired comparison [$t(5) = 4.45$, $P = 0.0043$] (Fig. 1C). Sensory clusters also showed significantly less accommodation 3 days [$t(5) = 7.20$, $P = 0.0008$], 6 days [$t(4) = 6.45$, $P = 0.003$], 7 days [$t(3) = 6.36$, $P = 0.008$], and 10 days [$t(3) = 3.76$, $P = 0.035$] after crushing the ipsilateral pedal nerve than before (Fig. 1D). Our paired testing procedure (*10*) and the number of sensory neuron pairs (five to eight) examined within each animal also allowed comparisons of cell populations in individual animals. Sensory neurons with crushed axons displayed less action potential accommoda-

Table 1. Electrophysiological properties altered by axon crush. Data are expressed as mean ± SEM, except for the percentage of cells showing afterdischarge. Values of P were determined with two-tailed paired t tests (n.s., not significant), except for the incidence of afterdischarge (Fisher's exact test). The term AHP denotes afterhyperpolarization. The term n indicates pairs of sensory clusters (one per animal) except in the afterdischarge incidence, where it refers to the total number of sensory neurons in crushed axon and control clusters in the subset of animals ($n = 5$) that displayed afterdischarge during determination of spike threshold. Each EPSP data point was the average of the ten largest EPSPs recorded in the motor neuron from one sensory cluster.

Property	Control	Crushed axon	P	n
Accommodation (number of spikes)	5.9 ± 0.9	14.5 ± 1.0	0.001	26
Threshold (nA)	1.39 ± 0.05	1.30 ± 0.07	0.005	26
AHP (mV)	5.8 ± 0.2	4.4 ± 0.3	0.001	26
Resting potential (mV)	44.1 ± 0.5	43.9 ± 0.5	n.s.	26
Spike amplitude (mV)	78.6 ± 1.5	79.3 ± 1.5	n.s.	26
Spike duration (ms)	1.9 ± 0.2	2.4 ± 0.2	0.001	9
EPSP (mV)	4.5 ± 0.9	11.7 ± 2.3	0.01	8
Afterdischarge incidence	5%	33%	0.01	86

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tion in 21 of 26 animals tested 3 or more days after nerve crush, including the single animal tested at 23 days ($P < 0.05$ in each case).

No significant differences in action potential accommodation were observed between clusters ($n = 4$ pairs) or cells ($n = 28$ pairs) 1 day after pedal nerve crush (Fig. 1D). To see if this lack of change might be due to inadequate time for signals from the distant (15 to 30 mm) site of injury to be carried by retrograde axonal transport to the sensory somata, we produced the injury within 1 mm of the somata by crushing one of the pedal-pleural connectives. Two of three animals showed a significant decrease in action potential accommodation in sensory neurons subjected to nearby axon crush 1 day earlier ($P < 0.02$ and $n = 7$ cell pairs in each case). No differences were seen 1 hour after crush (*11*).

In another study crushing nerves or connectives that did not contain axons of the tested sensory neurons in various combinations failed to decrease accommodation (*11*). This axonal specificity and the long latency of the soma plasticity indicated that injury of the axon of the sensory neuron is important and that the inducing signals are conveyed by retrograde axonal transport. These features suggested that neuromodulator release from interneurons (*12*) was not necessary for the enhanced excitability.

To minimize any involvement of synaptically released neuromodulators, we crushed the nerves under conditions in which synaptic release and spike activity were severely limited. We accomplished this by injecting the animal with ice-cold isotonic $MgCl_2$ solution and crushing the nerves and connectives while the temperature was 1° to 2°C. This procedure largely blocked synaptic transmission, greatly reduced the number of sensory action potentials elicited by nerve crush, and prevented changes in behavioral state after surgery (*13*). Eight animals were examined 2 to 12 days after unilateral axon crush under these conditions. The clusters subjected to axon crush responded with more action potentials during the accommodation test than did contralateral clusters [9.9 ± 1.2 and 1.5 ± 0.1 spikes, respectively; $t(7) = 7.77$, $P = 0.0001$]. Moreover, within each animal the cells with crushed axons showed less accommodation than did control cells ($P < 0.05$ in each case).

Other properties of the sensory neurons were altered 3 to 23 days after axon injury (Fig. 2 and Table 1). Before testing accommodation, we determined action potential threshold by injecting into the soma a graded series of 20-ms depolarizing pulses. The amount of current required to initiate an action potential was significantly lower in cells with crushed axons than in control cells, as was the size of the afterhyperpolarization. Action potential duration was significantly enhanced by injury (Fig. 2B and Table 1). Five animals displayed afterdischarge (Fig. 2C) in one or more sensory neurons during testing. In these animals, 14 of 43 cells subjected to axon crush displayed afterdischarge, whereas only 2 of 43 control cells did (Table 1). No significant differences were seen in resting potential or action potential amplitude.

We also examined the effects of nerve crush on synaptic connections from pleural sensory neurons to ipsilateral pedal motor neurons (*14*). We surveyed connections to each motor neuron from 15 to 20 sensory neurons in the ipsilateral VC cluster, using intracellular stimulation to activate each sensory neuron. The mean of the ten largest excitatory postsynaptic potentials (EPSPs, one per sensory neuron) with short latency

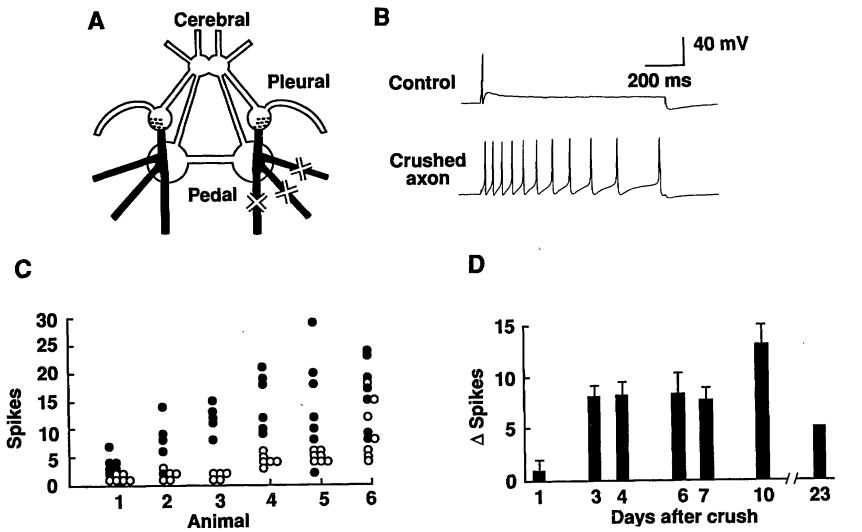

Fig. 1. Long-term alteration of action potential accommodation induced by nerve crush. (**A**) Sensory neuron somata (dots) are located in each pleural ganglion and project their axons (dark lines) through the ipsilateral pedal ganglion into pedal nerves. The X's indicate crush of pedal nerves 15 to 30 mm from pleural somata. In some experiments the connective between the pleural and pedal ganglia was crushed on one side. (**B**) Action potential (spike) accommodation in a pair of sensory neurons during 1-s depolarizing test pulses 4 days after unilateral pedal nerve crush. Accommodation is defined as a decline in spike frequency during the pulse. (**C**) Number of action potentials evoked by 1-s test pulses 4 days after unilateral pedal nerve crush. Each point (●, crushed axon; ○, control) represents the response of a single sensory neuron. (**D**) Mean (± SEM) differences between the number of spikes evoked by the 1-s test pulses in sensory clusters subjected to axon crush and those evoked in contralateral control clusters. The number of spikes evoked in each cell within a cluster was averaged, yielding a pair of spike scores for each animal. These scores were then averaged across each group of animals (one group per test interval), and the mean difference was plotted at each test interval.

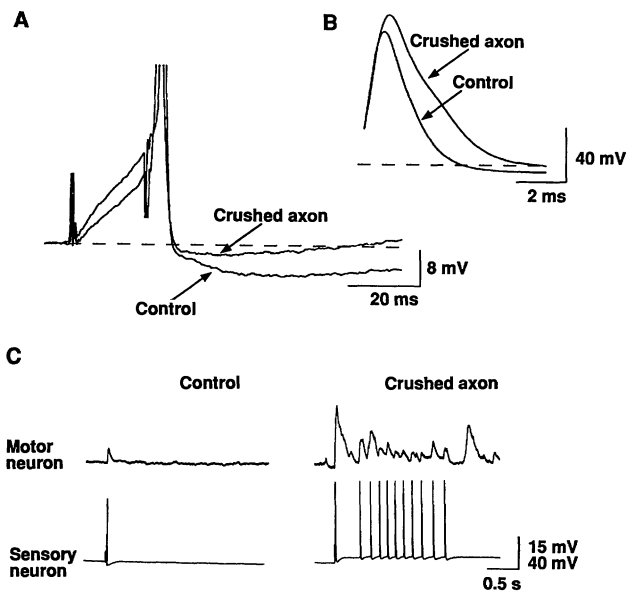

Fig. 2. Long-term alteration of other electrophysiological properties. (**A**) Superposition of threshold responses from a pair of sensory neurons, illustrating the difference in afterhyperpolarization. (**B**) Superposition of threshold action potentials from another pair of sensory neurons, illustrating the difference in spike duration. (**C**) Example of the largest EPSPs evoked by brief stimulation of control and crushed axon sensory neurons from one animal. This record also shows an afterdischarge after the 20-ms stimulus in the crushed axon sensory neuron. The scale for the y axis differs for the two neurons; the top and bottom values correspond, respectively, to the motor and sensory neuron.

in each motor neuron was significantly larger on the side in which the sensory axons were crushed than that on the control side (Fig. 2C and Table 1).

These results indicate that axonal injury and aversive learning induce similar long-term changes in *Aplysia* sensory neurons: decreased action potential accommodation (*7, 15*), decreased threshold (*2, 15*), decreased afterhyperpolarization (*7*), increased action potential duration (*16, 17*), synaptic facilitation (*2, 16–18*), and increased probability of afterdischarge (*2*). Because spike broadening in *Aplysia* sensory neurons is associated with presynaptic facilitation (*16*), the synaptic facilitation observed after axonal injury may involve enhancement of transmitter release (*19*). All of these alterations act to increase the signaling effectiveness of the sensory neuron.

However, sensory plasticity after axonal injury involves signals that are distinct from signals implicated in learning, that is, neuromodulators released from interneurons (*12*) and action potential activity in sensory neurons (*2, 20*). First, learning-induced plasticity reaches a maximum value in less than a day (often within minutes to hours) (*2, 17, 20*), whereas plasticity after nerve crush may take several days to become maximal, depending on how far the site of axonal injury is from the soma (*11*). Second, the alterations produced by nerve crush are as large or larger than learning-associated alterations in these cells (*2, 17, 18, 20*), even though they are induced under conditions in which rapid neural signaling is severely reduced. The injury signals may be generated directly by trauma of sensory neuron axons, perhaps by an interruption of retrograde transport of trophic factors (*3*). Slow axonal signals might also arise from interactions of the axon with extracellular substances produced by nearby tissue damage or from hemocytes attracted to the site of injury (*21*).

Although some of the induction signals differ, the qualitative similarity of mechanosensory plasticity associated with classical conditioning, general sensitization, site-specific sensitization around a wound, and axon injury in *Aplysia* suggests that a common cellular program for long-term sensory facilitation might be induced by each procedure, and supports the hypothesis that some memory mechanisms evolved from primitive adaptive responses to axonal injury (*4*).

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8. *Aplysia californica* (100 to 250 g) were anesthetized by injection of an isotonic $MgCl_2$ solution, equal to 35% of the body volume, into the neck. Each pedal nerve was pinched with thin forceps at two sites separated by 5 mm. Microscopic inspection confirmed nerve injury and indicated that the axons were often completely transected within the surrounding sheath.
9. We separated the effects on threshold from effects on spike accommodation by first determining the threshold with 20-ms depolarizing pulses and then applying a 1-s pulse at a constant current level 25% greater than the 20-ms threshold current for that cell.
10. We impaled and examined sequentially cells in isolated pairs of pedal-pleural ganglia, alternating between the left and right pleural ganglia. We paired cells within the same animal by correlating locations within each sensory cluster, and the same microelectrode was used for both. Statistical comparisons within groups of animals were made with two-tailed *t* tests, with average scores per cluster as data points. Subsequent comparisons of cell pairs in individual animals were performed with one-tailed Wilcoxon tests.
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13. We accelerated recovery from anesthesia by warming the animal to 25°C and injecting one to two times its volume of seawater. The return of normal behavior, including spontaneous locomotion and feeding, within 1 to 2 hours of seawater injection indicated that insufficient sensory activation or release of neuromodulators had occurred in response to nerve crush or surgery to cause long-term sensitization (E. T. Walters and A. L. Clatworthy, unpublished observations).
14. Crushes were performed at 1° to 2°C after injection of $MgCl_2$ solution. Motor neurons were matched by location, physiological properties, antidromic responses to nerve stimulation, and regions within the VC cluster giving maximal synaptic inputs. EPSP latencies were 5 to 8 ms, indicating that they were probably monosynaptic [see (*6*)].
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