Published in final edited form as:

Neuroscience. 2007 December 19; 150(4): 915–925.

Lasting changes in a network of interneurons after synapse regeneration and delayed recovery of sensitization

Albert K. Urazaev¹, Sara Arganda², Kenneth J. Muller^{3,*}, and Christie L. Sahley¹

¹ Department of Biological Sciences, Purdue University, West Lafayette, IN 47907

² Department of Theoretical Physics, Universidad Autonoma de Madrid, Madrid, Spain

³ Department of Physiology & Biophysics and Neuroscience Program, University of Miami School of Medicine, Miami, FL 33101

Abstract

Regeneration of neuronal circuits cannot be successful without restoration of full function, including recovery of behavioral plasticity, which we have found is delayed after regeneration of specific synapses. Experiments were designed to measure neuronal changes that may underlie recovery of function. Sensitization of the leech withdrawal reflex is a non-associative form of learning that depends on the S-interneuron. Cutting an S-cell axon in Faivre's nerve disrupted the capacity for sensitization. The S-cell axon regenerated its electrical synapse with its homologous cell after 3 to 4 weeks, but the capacity for sensitization was delayed for an additional 2 to 3 weeks. In the present experiments another form of non-associative conditioning, dishabituation, was also eliminated by S-cell axotomy; it returned following regeneration. Semi-intact preparations were made for behavioral studies, and chains of ganglia with some skin were used for intracellular recording and skin stimulation. In both preparations there was a similar time-course, during 6 weeks, of a lesion induced decrease and delayed restoration of both S cell action potential threshold to depolarizing pulses and S-cell firing in response to test stimuli. However, the ability of sensitizing stimuli to decrease S-cell threshold and enhance S-cell activity in response to test stimuli did not fully return after regeneration, indicating that there were lasting changes in the circuit extending beyond the period necessary for full recovery of behavior. Intracellular recordings from the axotomized S-cell revealed a shift in the usual balance of excitatory and inhibitory input, with inhibition enhanced. These results indicate that loss of behavioral plasticity of reflexive shortening following axotomy in the S-cell chain may be related to reduced S-cell activity, and that additional processes underlie full recovery of sensitization of the whole body shortening reflex.

Keywords

Leech; learning; plasticity; reflex; axotomy; repair

Introduction

For full recovery from damage to the nervous system it has been thought since the time of Cajal that severed axons and disrupted synapses must regenerate (Ramón y Cajal, 1928), but reliably achieving this continues to be a major problem (Houle and Tessler, 2003; Dobkin, 2007).

*Corresponding author. Tel.: +1-305-243 3364; fax: +1-305-243243 5931 E-mail address: kmuller@miami.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Although accurate repair is challenging in the mammalian CNS, it is strikingly successful in the CNS of the leech (Nicholls, 1987). Yet even in the leech it has been found that recovery of a simple form of learning, sensitization of reflexive shortening, is delayed for weeks after precise regeneration of a single key axon and its synapse (Burrell et al., 2003). The delay is presumably because during the period of denervation, changes in neurons and their connections occur that must be reset once regeneration is complete, but this hypothesis has not been tested.

In diverse species axotomy can produce a cascade of changes both within the injured neuron and trans-synaptically in other neurons in central pathways. Axotomized motoneurons, for example, undergo a characteristic sequence of electrophysiological and structural changes altering their synaptic input and may not recover until after each severed axon regenerates its synaptic connections with denervated muscle (Blinzinger and Kreutzberg, 1968; Kuno and Llinás, 1970a; Kuno and Llinás, 1970b). Interestingly, those changes may be quite different for adults and neonates (Mentis et al., 2007). In addition to retrograde changes affecting the axotomized neuron's dendritic inputs, there may be a cascade of orthograde changes within central circuits, including degenerative changes (Cowan, 1970), which have also been identified in the leech (Jansen et al., 1974). Functional changes may be a consequence of alterations in neuronal structure (Dickson et al., 2007) and connectivity (Koerber et al., 2006), and they may involve alterations in molecular properties including of transmitter receptors (Eleore et al., 2005).

Because of the complex nature of recovery of behavior and particularly plastic behavior in mammals following CNS injury (Dobkin, 2007), it has been useful to study the link between regeneration of specific synapses and the recovery of simple learning in the leech. The system of S-interneurons in the leech, which receives sensory input and excites motoneurons involved in reflexive shortening, is crucial for sensitization of reflexive shortening, but it is evidently not required for shortening itself. Sensitization, or enhancement, of reflexive shortening in response to a weak test stimulus arises following a stronger, sensitizing stimulus which would be considered noxious. The responses to test stimuli themselves habituate, or decline, either without the sensitizing stimulus or if just one S-cell has been killed or had its axon cut (Sahley et al., 1994; Modney et al., 1997). Habituation reverses after a stimulus like that used to produce sensitization, a process called dishabituation. Dishabituation is reportedly only partially reduced by killing the S-cell in the same segment as the test stimulus (Sahley et al., 1994), but the effect on dishabituation of S-cell axotomy at a distance from the test stimulus has not been examined. Although the capacity for sensitization is restored after regeneration of the axon (Modney et al., 1997), it was found that the behavioral recovery was delayed by weeks following the return of high-frequency conduction of action potentials along the S-cell chain (Burrell et al., 2003). Experiments in the present paper were designed to reproduce these findings with a new preparation more suitable for electrophysiological recording and to determine whether changes in electrophysiological properties of the S-cells and their network could account for the delay in recovery. It was hypothesized that the system would reset to the original cellular and synaptic properties to restore full function.

Experimental procedures

Animals, surgery and recording

Adult *Hirudo* leeches, 3 g in body weight, were purchased from a commercial supplier (Leeches USA, Westbury, NY) and maintained together in artificial pond water, pH 7.4, at 20°C. After animals were anesthetized in 8% ethanol in pond water, the ventral sinus was opened and the small bundle of ~100 axons containing the S-interneuron axon (Faivre's nerve) was cut using the tip of a 26 gauge hypodermic needle as described (Modney et al., 1997), except that for greater separation of stimulation sites the lesion was anterior to ganglion 9 rather than ganglion 7. An initial series of experiments in which Lucifer Yellow dye (5% in 0.1 M LiCl) was injected

into the lesioned S-cell body or its axon, to label the cell and show the extent of its processes and electrical connections, confirmed earlier work showing that the surgical procedure cut the S-cell axon. Leeches were then randomly divided into groups and allowed to recover for varying time periods from 1 to 6 weeks.

In preparation for dissection and experiments, animals were anesthetized at 4°C in ice-cold pond water. A schematic representation of the experimental preparation is presented in Fig. 1. For behavioral experiments, the preparation included the head and initial 11 segments intact to measure reflexive shortening via a tension transducer; ganglia 12–13 with associated segmental nerves (roots) were exposed. The preparation was pinned to a layer of cured Sylgard 184 silicone rubber in a dish filled with leech physiological saline (in mM: NaCl, 110; KCl, 4; CaCl₂, 1.8; HEPES, 10; pH 7.4). A monofilament nylon suture thread (Ethilon, Ethicon Inc., Somerville, NJ) connected the anterior sucker to a force transducer (Astromed/Grass Instruments, West Warwick, RI) to measure the magnitude of the reflexive whole-body shortening response. Action potentials were recorded with a suction electrode attached to the connectives posterior to ganglion 13. Test and sensitizing stimuli were delivered through fine Teflon-coated silver wires (Medwire, Mount Vernon, NY) attached to the skin in segments 4–5 and 10, respectively, and bared at the skin surface (Fig. 1A).

Behavioral training (sensitization of the shortening reflex) was done by the procedures described previously (Sahley et al., 1994; Burrell et al., 2003). In brief, the test stimulus was a 3 ms square pulse at 1 V above the threshold level for inducing whole body shortening, while the sensitizing stimulus (2 trains of 10 pulses per train) was 3 V above the same threshold value. At the conclusion of behavioral experiments and during electrophysiological experiments, the propagation of action potentials along S-cell chain was measured with intracellular recording in combination with extracellular recording and stimulation to determine whether the severed axon had regenerated its connection with the adjacent S-cell from ganglion 8 (Fig. 2).

For microelectrode recordings, a ventral nerve cord from segments 3 through 12 was isolated, the blood sinus removed and suction electrodes applied to both ends of the nerve cord. A pair of silver wires was attached to the skin of segment 4 to deliver test stimuli and another pair attached to deliver sensitizing stimuli to segment 11 (Fig. 1B). Microelectrode recordings were made variously from the S-interneuron in ganglion 9 (S9), from the S-cell in ganglion 4 (S4), and in some experiments from the touch (T) and pressure (P) sensory neurons in ganglion 4. For intracellular recording from S4, the skin remained attached to one side, to which the test stimulation was given. Standard thin-walled glass microelectrodes (30-30-0, Frederick-Haer, Bowdoinham, ME) with tip resistance of 15–25 MΩ when filled with 3 M potassium acetate and high-impedance negative-capacitance amplifiers were used for both registering membrane potentials and current injections. Excitability of the S-interneuron was measured either by determining the current that elicited a single action potential. Intracellular signals were filtered with a low-pass 4302 Dual 24dB/octave filter (Ithaco, Ithaca, NY) and amplified using an AxoClamp 2B amplifier (Axon Instruments, Union City, CA). Extracellularly recorded signals were amplified with a P5 AC-coupled preamplifier (Grass-Telefactor, West Warwick, RI). To inject current, we used a 20 ms current pulse at the amplitude that elicited a single action potential within the first 10–15ms of the pulse. This amplitude of 20 ms pulse was considered as threshold amplitude and used as a measure of each cell's excitability. Another measure of cell excitability was the number of action potentials generated by 200ms depolarizing pulse (averaged over three consecutive pulses) at the same amplitude as a threshold for a 20 ms pulse (Burrell et al., 2001). A 200 ms hyperpolarizing pulse was used to monitor the S-cell input resistance. A dual-output S88 stimulator (Grass-Telefactor) with SIU5 stimulus isolation units (Grass-Telefactor) were used for intra- and extracellular stimulations.

Data were digitized using a Digidata 1322A interface and collected with Axoscope9 (Axon Instruments). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO).

Statistics

Repeated-measures Analysis of Variance (ANOVA) (behavioral data, thresholds; number of action potentials generated during 200 ms depolarizing pulses) or Repeated-measures Poisson Regression (number of action potentials (Littell et al., 1996)) were used for the analysis of differences between independent and dependent variables and a Least Squares Test was used for post-hoc analyses of significant main effects and interactions (SAS 9.1.3, SAS Institute, Cary, NC). Paired t-test was used to compare the effect of noxious stimulation on habituating animals and Chi-Square test for independence was used to determine an association between the variables in experimental groups and either time after axotomy or incidence of subthreshold synaptic responses to the posterior connective stimulation (InStat, GraphPad Software, San Diego, CA). The differences were considered significant if $p \leq 0.05$. Data are expressed as mean $\pm$ S.E.M. The mean number $\pm$ S.E.M. of S-cell action potentials was determined as an exponential (e^x) of the Least Squares Mean (x) $\pm$ S.E.M. calculated during a Poisson Regression analysis of experimental data.

Results

S-cell axotomy disrupts capacity for sensitization, which is delayed after regeneration

As a first step toward determining possible mechanisms underlying delayed recovery of the capacity for sensitization following regeneration of the S-cell axon, measurements were made to determine whether the phenomenon of recovery with a delay was replicable following the surgical axotomy used in initial studies of S-interneuron regeneration (Modney et al., 1997). Sensitization training caused an increase of touch-elicited whole body shortening in sham-operated animals (Figure 3A, filled circles). In contrast, leeches in the habituation control group, which did not experience the sensitizing stimulation, showed a decrease in responding to the repeated touch stimulus, thus they habituated (open circles; group effect, $F(1,4)=6.03$, $p<0.05$). Strong stimulation like the sensitizing stimulus but delivered following the repeated test stimuli reversed the effect of habituation (dishabituation) on the whole body shortening reflex (paired t-test, $p<0.05$), as previously reported (Sahley et al., 1994; Burrell et al., 2001).

The process of habituation to repetitive stimulation of skin is a common occurrence even for animals that have experienced sensitizing training (Burrell et al., 2001). For that reason, a decrease in response to the test stimulation due to habituation may be accounted for when assessing the behavioral responses in experimental groups (sensitizing training). The correction for the decline due to habituation results in the Mean Normalized Sensitization (MNS), which was the difference between the experimental response amplitude and that of habituation control leeches, each averaged for the corresponding block and calculated as a percentage of baseline. The MNS showed the net augmentation resulting from sensitization in sham-operated animals (Fig. 3A, filled squares).

In contrast, severing the S-cell axon by cutting Faivre's nerve eliminated the ability of leeches to show sensitization of the reflex, as previously reported (Modney et al., 1997). Similar experimental protocols and MNS calculations were made for the leeches 2 weeks (Fig. 3B), 3 weeks (data not shown), and 6 weeks (Fig. 3C) following axotomy. The MNS for each of these experimental groups is shown in Panel D. This normalization reveals that 2- and 3-week axotomized animals completely lost their capacity for sensitization, which fully recovered 6 weeks after the axotomy.

As shown in Fig. 3A–C, a similar sequence involving loss and restoration of dishabituation occurred. Although some loss of the capacity for dishabituation had been previously reported following injury, the loss was incomplete (Sahley et al., 1994); in those studies the sensitizing and dishabituating stimuli were more anterior, in the same segment as the test stimulus.

An alternative way to analyze the sensitization data was to compare the behavior of those animals during the recovery period according to whether conduction of impulses along the S-interneuron chain had been restored and whether the capacity for sensitization of reflexive shortening had returned. Fig. 4 shows, as in Fig. 3, the responses of animals in the Sham-operated group. In addition, animals tested during the period of recovery, 7–42 days after axotomy, were divided into 3 groups: the Lesion group, in which no physiological connection between the S-cells on either side of the lesion had occurred, the Intermediate group, in which a physiological connection with impulse conduction between S-cells was re-established, but the capacity for sensitization had not returned, and the Recovery group, in which both conduction and capacity for sensitization had returned.

The Repeated-measures ANOVA revealed a significant effect of treatment group ($F(3,185) = 21.85$; $p < 0.001$), but no significant effect of trial block ($F(4,185) = 0.41$; $p = 0.80$). However, the analysis did reveal a significant group*trial interaction ($F(12,185) = 1.88$; $p < 0.05$). A Least Squares Means post-hoc analysis of the group*trial interaction demonstrated that as a result of sensitization training, only animals in the Sham and Recovery groups showed a significant increase in responding. For Sham animals the significant increase in responding was evident in Block 1 and remained elevated throughout subsequent training (Sham-baseline vs blocks 1 to 4, $p < 0.01$). The sensitization-induced increase in responding for animals in the Recovery group did not appear until block 2 and then declined by blocks 3 and 4 (Recovery-baseline vs block 2, $p < 0.02$; baseline vs other blocks not different; Fig. 4).

These results were consistent with previous work (Burrell et al., 2003) showing that cutting the axon of a single S-cell disrupts the capacity for sensitization of whole-body shortening and that the capacity returns after a delay of several weeks. The behavioral recovery was not coincident with the reconnection of the S-cell with its synaptic target (3–5 weeks following axotomy), the axon of the single S-cell in the adjacent ganglion, but rather occurs at some later time. Fig. 5 shows that the number of animals falling into the Lesion category decreased across time (100% at 1 week, 70% at 2–3 weeks, 16.7% at 4–5 weeks and 0% at 6 weeks), whereas the number of animals in the Recovery category increased over time (0% at 1 week, 0% at 2–3 weeks, 25% at 4–5 weeks and 100% at 6 weeks). Significantly, leeches falling into the Intermediate category did not appear until 2–3 weeks following axotomy, increased at 4–5 weeks following axotomy and were no longer observed at 6 weeks. The correlation between time following injury and the extent of recovery indicates that time can be used in conjunction with electrophysiology to predict likelihood of behavioral recovery of the preparation (see below).

Behavioral training, with or without axotomy, does not change sensory neuron excitability or response to skin stimulation

In order to locate changes in the S-cell circuitry that might underlie changes in the reflex associated with behavioral sensitization, an important step was to determine whether sensitization affected mechanosensory neuron firing in response to cutaneous stimulation and excitability, and whether there was a change following surgery. To address this, the excitability of sensory T- and P-cells in midbody segmental ganglion 4 (Table 1) and their responses to test stimulation of the skin of that segment (Table 2) were measured in both control and axotomized animals that underwent sensitizing training.

Neither the threshold for the activation of sensory T- and P-neurons by a 20 ms depolarizing pulse nor the number of action potentials generated by a 200 ms depolarizing pulse was affected by sensitizing training (ANOVA for threshold: group effect $F(5,44)=0.02$, $p=1.00$ [Table 1], trial effect $F(5,172)=2.01$, $p=0.08$; group*trial interaction $F(25,172)=1.05$; $p=0.41$; Poisson Regression for action potentials: group effect $F(5,48)=1.10$, $p=0.37$, trial effect $F(3,126)=2.35$, $p=0.08$, group*trial interaction $F(15,126)=1.54$, $p=0.10$, data not shown). In addition, the number of action potentials produced in the T- and P-neurons in response to skin stimulation was not affected by sensitizing training (Poisson Regression: group effect $F(5,108)=2.09$, $p=0.07$ (Table 2), trial effect $F(3,108)=0.11$, $p=0.95$; group*trial interaction $F(15,108)=0.79$, $p=0.69$, data not shown). These results indicate that the changes in behavior and in S-cell threshold and reflexive firing associated with axotomy and repair cannot be simply explained by changes in sensory neuron excitability and responsiveness.

Axotomy eliminates the ability of sensitization to increase S-cell excitability

Earlier work showing that S-cell axotomy eliminates the capacity for sensitization (Burrell et al., 2003) and that sensitizing stimuli increase S-cell excitability (Burrell et al., 2001) raised the question of whether axotomy of one neuron in the chain of S-cells would eliminate the usual increase in excitability following sensitization. After animals were operated and allowed time to recover, they were partially dissected as a reduced preparation (Fig. 1B). This was used to measure excitability both as the threshold in response to a 20 ms pulse of depolarizing current injected into the soma and as the number of action potentials produced by a test stimulus. Identical measurements were performed in both the S9, whose axon was severed, and in the distant S4. S4 was of particular interest since it received the greatest direct input from the sensory neurons responding to the test stimulation of the skin. The numbers of impulses were measured intracellularly and confirmed with suction electrodes.

Fig. 6 shows that the number of baseline action potentials, i.e. those in response to a test stimulus prior to delivering the sensitizing stimuli, was not significantly different among the groups (Group effect for only baseline measurements by Poisson Regression: $F(3,37)=1.34$, $p=0.28$). For these measurements, intracellular recordings for the Lesion group were made in S4, since it was disconnected from S9, whereas after regeneration of S9 in the Early Regen (i.e. early regeneration) group S9 was used directly, because its activity occurred one for one with S-cell activity in the anterior suction electrode. For the Sham and Late Regeneration groups, activity in either or both S-cells, S4 and S9, was used in conjunction with anterior connective recordings and S4 and S9 activity were not different. The Repeated-measures Poisson Regression revealed a significant group*trial interaction ($F(12,170)=1.87$, $p=0.04$) but no significant effect of treatment group ($F(3,170)=1.06$, $p=0.37$) nor significant trial effect ($F(4,170)=0.57$, $p=0.69$). The Least Squares Means post hoc test for the group*trial interaction indicated that after a sensitizing stimulus, the number of S-cell action potentials produced by a test stimulus increases significantly in Blocks 1–3 in Sham animals ($p<0.05$), whereas in lesioned animals before regeneration (Lesion) the number dropped significantly in Blocks 1 and 4 ($p<0.05$), which was not true following regeneration.

Fig. 7 shows that another measure of excitability, the threshold of S4, followed a similar pattern. There was no significant difference between baseline thresholds among the groups, although there were differences following the sensitizing stimulus. In fact, the Repeated-measures ANOVA revealed a significant group*trial interaction ($F(15,186)=1.88$, $p=0.028$) but no significant effect of treatment group ($F(93,186)=2.4$, $p=0.07$) or significant trial effect ($F(5,186)=0.67$, $p=0.65$). The Least Squares Means post hoc test of group*trial interaction demonstrated that whereas in Sham controls the threshold dropped significantly in some time blocks following a sensitizing stimulus, this was not true of the axotomized preparations, including the Late Regen (i.e. late regeneration) preparations. In shams, the decrease in

threshold was evident during first two intervals, 1st–9th min and 13th–17th min, following sensitizing stimulation ($p < 0.05$), suggesting that the latter increased the S-cell excitability. Sensitizing stimulus did not change the threshold for S4 activation in both Lesion and Early Regeneration groups ($p > 0.05$). Unexpectedly, a decrease in S4 threshold seen in sham-operated leeches did not recover in the Late Regeneration group. In contrast, the action potential threshold significantly increased during 13th–17th min ($p < 0.01$) and 21st–25th min ($p < 0.05$) intervals after sensitizing stimulation, indicating a decrease in the S-cell excitability.

As another measure of excitability, the number of action potentials generated in the S-cell in ganglion 4 during a 200 ms depolarizing current pulse at an amplitude equal to the threshold amplitude for a 20 ms pulse was also monitored in these experiments. A Repeated-measures ANOVA of these data did not reveal significant group or trial effects ($F(3, 162) = 1.54$; $p = 0.21$ or $F(4, 162) = 0.86$; $p = 0.49$, respectively) nor did it reveal a group*trial interaction ($F(12, 162) = 0.79$; $p = 0.66$). These results suggest that neither sensitization training nor axotomy were effective in changing the second measure of excitability, namely the number of action potentials generated by a 200 ms depolarizing pulse (Suppl. Fig. 1).

Taken together, the results on changes in S-cell excitability were consistent with earlier findings. Sensitizing stimuli in sham-operated animals, like unoperated animals reported previously, increased excitability in parallel with the enhanced shortening indicating behavioral sensitization. Following axotomy, which eliminated the capacity for behavioral sensitization, both measures of excitability showed that sensitizing stimuli that had previously enhanced excitability no longer did so. This situation continued after regeneration of the S-cell axon and restoration of conduction even at high firing frequencies along the chain of S-cells, consistent with continued absence of the capacity for sensitization of reflexive shortening. Surprisingly, however, there was no recovery of S-cell excitability by either measure for the Late Regenerates, those that by 6 weeks had recovered the capacity for sensitization.

S-cell action potentials at rates observed during sensitizing training do not change S-cell excitability

Noxious stimulation of the skin during sensitizing training causes bursts of action potentials propagating along the S-cell chain. Does this elevated activity in the S-cells affect their excitability? In the present experiments, the average number of S-cell action potentials generated during 2 sensitizing trains was 24.4 ± 2.2 spikes ($n = 40$) and was not different between experimental groups ($p > 0.05$; data not shown). To determine whether these spikes could affect the excitability in S-cell, the excitability was measured in S9 before and after a burst of spikes 1–1.5 hour after completion of the behavioral part of the experiment. Accordingly, a 800 ms depolarizing pulse (at a threshold amplitude for the 20 ms pulse) was passed through the cell membrane, generating a burst of 28 ± 3.5 spikes ($n = 23$; $p > 0.05$ compared to the burst during sensitizing trains). This burst did not change the threshold for S-cell activation by a 20 ms pulse, nor did it change the number of action potentials generated during 200 ms depolarizing pulse ($p > 0.05$; data not shown). This indicates that the changes in S-cell excitability following axotomy, reported in the previous section, are a consequence of the activity in cells in addition to the S-cell during the sensitizing stimulus, since activity in the S-cell by itself is insufficient to cause measurable change. It does not indicate whether activity in the S-cell itself is necessary for the change in excitability, although other experiments involving selective axotomy indicate that S-cell activity is probably essential (Burrell et al., 2003).

Synaptic activation of the axotomized S-cell and distant changes in threshold

Stimulation of and recording from the connectives, as in Fig. 1B, was used not only to determine whether the severed axon of S9 had regenerated its synaptic connection with the neighboring S8, but also to measure the relative excitability of distant S-cell axons and to activate synaptic

input to the S-cell. In intact preparations, anterior or posterior connective stimulation directly activated the S-cell at the lowest threshold of any axon in the connectives, eliciting a rapidly conducting action potential that arrived with short latency at the intracellular microelectrode in S9 (Figs. 2A and 8A, left) and at the extracellular suction electrode.

There was no directly driven synaptic activity observed in the S-cells caudal to the lesion in response to anterior connective stimulation at threshold levels (Fig. 2B). Axotomy interrupted action potential propagation along the S-cell chain. However, increasing the amplitude of connective stimulation could synaptically activate the S-cell by recruiting axons of additional neurons having synaptic connections with the S-cell (Fig. 2B). Reconnection of the severed S-cell axon with its target restored low-threshold direct activation of the S-cell by through conduction (Fig. 2D), although in one of 13 experiments in Early Regeneration group we found an early stage at which through conduction appeared to be intermittent (Fig. 2C).

Stimulation of the connectives posterior to ganglion 12, 3 segments posterior to the axotomized neuron, showed that the threshold of the S-cell axon rose relative to other axons in the same segment of nerve cord. Although in absolute terms the threshold of the S-cell did not appear to change in the axotomized preparation, during simultaneous intracellular recording from S9 and suction electrode 1 recording from the anterior connective (Fig. 1B) in both the Early and Late Regeneration groups, other fibers were recruited before the S-cell axon was. This rise in relative threshold for the S-cell axon became evident when we observed synaptic potentials in S9 during stimulation of the posterior connective at or below threshold. As shown in Fig. 8, there were EPSPs alone (Fig. 8A, *middle*) and EPSPs followed by IPSPs, a biphasic response (Fig. 8A, *right*). In the preparations with a biphasic synaptic response, a gradual increase of the amplitude of stimulation from zero initially evoked an appearance of EPSP that was typically followed by IPSP (Fig. 8B, *left*). As shown in Fig. 8B, *right*, the IPSP amplitude changed in a manner consistent with a standard inhibitory conductance increase with a reversal potential of approximately -65 mV. When threshold for S-cell activation was reached, a directly activated action potential propagated along the S-cell chain, although occasionally a synaptically driven S-cell spike was observed at subthreshold amplitudes for the direct S-cell activation. Although driven synaptic activity was occasionally observed in sham-operated leeches, its incidence increased in axotomized animals, as shown in Fig. 9.

These results suggest that axotomy caused changes in the activity of the neurons having synaptic connections with the S-cell. These changes may have involved both plasticity in existing synapses and appearance of new synaptic contacts, including inhibitory connections. The appearance of inhibitory synapses may have contributed to a decreased excitability of the S-cells following axotomy and their decreased response to the test stimulation of the skin. This could be a part of the mechanism of the loss of the ability for sensitization after S-cell axotomy.

Discussion

The experiments presented here have extended previous results from laser and surgical axotomy, confirming that when the S-cell axon regenerates its electrical synapse with its usual target, the S-cell in the next ganglion, the animal's capacity for sensitization of reflexive whole-body shortening is restored. They also confirmed the puzzling delay of days to weeks following reconnection for recovery of behavior. The results of surgical axotomy in which Faivre's nerve was cut resembled those following laser axotomy of the S-cell alone. Moreover, the behavioral measures showed a strong correlation between time after surgery and the recovery of behavior, so that there was little behavioral recovery until 6 weeks, whether the connection had regenerated or not.

This correlation made it possible to look for changes in the properties of identified neurons that might account for the behavior by using a reduced preparation even without simultaneous behavioral measures. The preparation permitted combined intracellular and extracellular recording during standard stimulation regimes used for training (Fig. 1B). It was found, for example, that sensory cell activity did not change in response to a sensitizing stimulus or following S-cell axotomy (Tables 1 and 2).

Because the S-cell chain must be intact to produce sensitization of reflexive shortening and because S-cells are recruited into the reflex following sensitization (Sahley et al., 1994; Modney et al., 1997; Burrell et al., 2003), it was expected that during the first 2 weeks after axotomy the sensitizing stimulus would not increase the S-cell's activity in response to a test stimulus, and this was confirmed (Fig. 6). In fact, for some time blocks the response declined, consistent with elimination of capacity for sensitization. In agreement with the delay in recovery of behavior associated with the period of intermediate recovery at 3 to 5 weeks when the synaptic connection between S-cells had been re-established and through-conduction at high frequencies had been restored, the sensitizing stimulus did not increase the number of S-cell impulses generated in response to a test stimulus. The response was in some respects midway between the Sham and the Lesioned preparations, with no significant change in response to the sensitizing stimulus. But for the Late Regenerated group there was still no return to the Sham condition in which S-cell activity following sensitization rose in response to the test stimulus. This suggests that although the S-cell is required for the restoration of sensitization, its activity cannot account for the enhanced shortening in the sensitized state following regeneration of the S-cell axon. It is not clear whether it is still involved in or required for the response to test stimuli. Our working hypothesis, that the network simply resets to its original state to restore full function, is evidently not tenable.

The changes in *excitability* of S4 were in general consistent with the results for S-cell activity in response to test stimuli delivered to segment 4 (Fig. 7). We had previously reported that as activity following the test stimulus rises in sensitized preparations (Modney et al., 1997; Cruz et al., 2007), so too does excitability as measured by reduced threshold (Burrell et al., 2001). But just as it was unexpected that in response to a test stimulus the activity in the S-cell chain would not rise in the sensitized preparation in the Late Regenerated preparation, it was not predicted that the threshold of S4 would rise at some time blocks in the same preparations. The conclusion again is that the role of the S-cell may be more important for the establishment of the sensitized state than in participating in the sensitized reflex during the late phase of recovery of behavior. It implies that one should look elsewhere for underlying change or changes in the circuit, and that the usual enhanced excitability of the S-cell may be missing or neutralized.

The S-cell is involved in a positive feedback circuit with the serotonergic Retzius cells, which are excited by and in turn excite the S-cell in each ganglion (Crisp and Muller, 2006). Moreover, serotonin applied to the S-cell increases its activity (Burrell et al., 2002) and mimics the effects of sensitization training, enhancing S cell excitability in a similar reduced but behaving preparation (Burrell et al., 2001). This provides a mechanism by which the S-cell is recruited into the shortening circuit. Although in the present experiments driven activity in the S-cell itself did not change its own excitability, this may not be indicative of the effects of S-cell activity in intact S-cell chains during sensitizing stimuli, which activate all classes of sensory neuron and therefore elicit simultaneous activity in many neurons that may then drive the Retzius cells, including through the S-cells themselves. It is nevertheless possible that following a lesion the positive feedback circuit with the Retzius cells is disrupted, or becomes changed with time, so that S-cell excitability is no longer enhanced by the sensitizing stimulus.

One mechanism that could neutralize the usual enhanced excitability of the S-cell is the increased inhibition of the S-cell that develops during the weeks following axotomy, as

illustrated in Figs. 8 and 9. The results are merely suggestive, since the connectives were stimulated as a whole, albeit at levels sub-threshold for the S-cell, which usually has the lowest threshold of any axons in the connectives. But IPSPs are not typically seen in S-cells, even when the strength of stimulation rises above the threshold for the S-cell. Furthermore, the baseline threshold for excitation of the S-cells in the chain, either to extracellular or intracellular stimulation, did not change following focal injury and with regeneration. Enhancement of inhibition and its gradual spread following injury has been reported for other neurons in the leech, where a qualitative shift of connections from excitatory to inhibitory can alter reflexive behavior (Jansen et al., 1974).

The result that axotomy of S9 eliminated the capacity for dishabituation was unexpected, since in an earlier study when the S-cell in ganglion 4 was ablated, dishabituation was reduced but not eliminated although the capacity for sensitization was abolished (Sahley et al., 1994). This had indicated that sensitization and dishabituation may operate by separate mechanisms. But the earlier study involved ablation of S4, when both the test and sensitizing stimuli were applied to segment 4 (Sahley et al., 1994). The earlier result showing that the capacity for dishabituation was partially lost and that for sensitization was completely lost indicated that separate mechanisms underlie dishabituation and sensitization. In the present study applying the strong sensitizing and dishabituating stimuli at segment 10, it appears that an intact S9 axon is required for both processes. While it will be important in the future to determine whether bypassing S9, stimulating strongly anterior to its segment, can produce both full sensitization and dishabituation, it also appears that S4 is only partially required for dishabituation, in contrast with its essential involvement in sensitization. These latest results speak directly to the issue of whether dishabituation and sensitization operate by separate mechanisms (Poon and Young, 2006), with evidence for their differences (Bristol et al., 2004; Hawkins et al., 2006; Asztalos et al., 2007), and indicate that their separation can depend on critical elements of the circuit.

The results reported here have significance for our general understanding the steps involved in the repair of disrupted circuits following axotomy and, in particular, understanding the mechanisms for restoration of plastic behavior and learning following injury. The S-cells in the leech are noteworthy not only because their regeneration of connections is highly reliable and has been tracked in detail, but also because the S-cell is key in two stages of sensitization of whole body shortening. The cells are instrumental in conveying the sensitizing signal along the nerve cord, and the S-interneurons, interposed between sensory and motor cells, are recruited into the whole body shortening circuit, their firing correlating with the strength of shortening in the sensitized state. In the adult mammalian CNS, where axon regeneration is limited, it has been known for some time that sprouting to form novel connections in central circuits may be required for recovery of function and to compensate for damage to axons (Gilbert and Wiesel, 1992; Jain et al., 1997; Florence et al., 1998; Koerber et al., 2006). It is also known that injury itself can trigger long lasting changes in pain circuits, whose sensory and behavioral consequences include supersensitivity to sensory inputs (Salter, 2004; Moss et al., 2007). These changes are of particular interest given the compelling links and shared mechanisms between nociception and plastic changes in behavior in both vertebrates and invertebrates (Walters and Ambron, 1995). While it would seem that precise regeneration of the original connections would be an effective, reliable means for restoring function, it is evident from experiments with the S-cell that the cell may not return to its original state. It is true that without regeneration of the S-cell axon, the capacity for sensitization of reflex shortening is not restored (Modney et al., 1997; Burrell et al., 2003), but once its connections are re-established, the S-cell does not recover its original response to sensitizing stimulation, at least within 6 weeks of axotomy. This may account for the delay in return of full function, as the S-cell circuit's renewed conduction along the chain gives rise to new central processes, not yet identified, that enhance reflexive shortening following sensitizing stimulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

Funded in part by a grant from the NIH/NINDS to KM and CS. SA was supported by the fellowship from Dirección General de Universidades e Investigación, Comunidad Autónoma de Madrid, Spain. We thank Dr. Jeff Lucas for the help with statistical analysis. We thank Jeff Lipitz, Stewart Samuels, Xianchong Zhou and Drs. Emmanuel Ngu and Georgina Cruz for their helpful comments, Adam Savitskas, Annie Kmetz, Katie Byrum, Nicky Weston and Lauren Mioton for their assistance with data analysis, and Rodney McPhail for Figure 1.

List of Abbreviations

ANOVA	Analysis of Variance
EPSP	excitatory postsynaptic potential
IPSP	inhibitory postsynaptic potential
MNS	mean normalized sensitization
S4	S-cell in ganglion #4
S9	S-cell in ganglion #9

Reference List

- Asztalos Z, Baba K, Yamamoto D, Tully T. The *fickle* mutation of a cytoplasmic tyrosine kinase effects sensitization but not dishabituation in *Drosophila melanogaster*. *J Neurogenet* 2007;21:59–71. [PubMed: 17464798]
- Blinzinger K, Kreutzberg G. Displacement of synaptic terminals from regenerating motoneurons by microglial cells. *Z Zellforsch Mikrosk Anat* 1968;85:145–157. [PubMed: 5706753]
- Bristol AS, Sutton MA, Carew TJ. Neural circuit of tail-elicited siphon withdrawal in *Aplysia*. I. Differential lateralization of sensitization and dishabituation. *J Neurophysiol* 2004;91:666–677. [PubMed: 13679401]
- Burrell BD, Sahley CL, Muller KJ. Non-associative learning and serotonin induce similar bi-directional changes in excitability of a neuron critical for learning in the medicinal leech. *J Neurosci* 2001;21:1401–1412. [PubMed: 11160412]
- Burrell BD, Sahley CL, Muller KJ. Differential effects of serotonin enhance activity of an electrically coupled neural network. *J Neurophysiol* 2002;87:2889–2895. [PubMed: 12037192]
- Burrell BD, Sahley CL, Muller KJ. Progressive recovery of learning during regeneration of a single synapse in the medicinal leech. *J Comp Neurol* 2003;457:67–74. [PubMed: 12541325]
- Cowan, WM. Anterograde and retrograde transneuronal degeneration in the central and peripheral nervous system. In: Nauta, WJH.; Ebessson, SOE., editors. *Contemporary Research Methods in Neuroanatomy*. New York: Springer-Verlag; 1970. p. 217–251.
- Crisp KM, Muller KJ. A 3-synapse positive feedback loop regulates the excitability of an interneuron critical for sensitization in the leech. *J Neurosci* 2006;26:3524–3531. [PubMed: 16571760]
- Cruz GE, Sahley CL, Muller KJ. Neuronal competition for action potential initiation sites in a circuit controlling simple learning. *Neuroscience*. 2007

- Dickson TC, Chung RS, McCormack GH, Staal JA, Vickers JC. Acute reactive and regenerative changes in mature cortical axons following injury. *Neuroreport* 2007;18:283–288. [PubMed: 17314672]
- Dobkin BH. Curiosity and cure: Translational research strategies for neural repair-mediated rehabilitation. *Dev Neurobiol.* 2007
- Eleore L, Vassias I, Vidal PP, de Waele C. Modulation of the glutamatergic receptors (AMPA and NMDA) and of glutamate vesicular transporter 2 in the rat facial nucleus after axotomy. *Neurosci* 2005;136:147–160.
- Florence SL, Taub HB, Kaas JH. Large-scale sprouting of cortical connections after peripheral injury in adult macaque monkeys. *Science* 1998;282:1117–1121. [PubMed: 9804549]
- Gilbert CD, Wiesel TN. Receptive field dynamics in adult primary visual cortex. *Nature* 1992;356:150–152. [PubMed: 1545866]
- Hawkins RD, Cohen TE, Kandel ER. Dishabituation in *Aplysia* can involve either reversal of habituation or superimposed sensitization. *Learn Mem* 2006;13:397–403. [PubMed: 16705138]
- Houle JD, Tessler A. Repair of chronic spinal cord injury. *Exp Neurol* 2003;182:247–260. [PubMed: 12895437]
- Jain N, Catania KC, Kaas JH. Deactivation and reactivation of somatosensory cortex after dorsal spinal cord injury. *Nature* 1997;386:495–498. [PubMed: 9087408]
- Jansen JKS, Muller KJ, Nicholls JG. Persistent modification of synaptic interactions between sensory and motor nerve cells following discrete lesions in the central nervous system of the leech. *J Physiol (Lond)* 1974;242:289–305. [PubMed: 4376167]
- Koerber HR, Mirnics K, Lawson JJ. Synaptic plasticity in the adult spinal dorsal horn: the appearance of new functional connections following peripheral nerve regeneration. *Exp Neurol* 2006;200:468–479. [PubMed: 16696973]
- Kuno M, Llinás R. Alterations of synaptic action on chromatolysed motoneurons of the cat. *J Physiol (Lond)* 1970a;210:823–828. [PubMed: 4322767]
- Kuno M, Llinás R. Enhancement of synaptic transmission by dendritic potentials in chromatolysed motoneurons of the cat. *J Physiol (Lond)* 1970b;210:807–821. [PubMed: 4322766]
- Littell, RC.; Milliken, GA.; Stroup, WW.; Wolfinger, RD. SAS Systems for mixed models. Cary NC: SAS Institute; 1996.
- Mentis GZ, Diaz E, Moran LB, Navarrete R. Early alterations in the electrophysiological properties of rat spinal motoneurons following neonatal axotomy. *J Physiol.* 2007
- Modney BK, Sahley CL, Muller KJ. Regeneration of a central synapse restores non-associative learning. *J Neurosci* 1997;17:6478–6482. [PubMed: 9236255]
- Moss A, Beggs S, Vega-Avelaira D, Costigan M, Hathway GJ, Salter MW, Fitzgerald M. Spinal microglia and neuropathic pain in young rats. *Pain* 2007;128:215–224. [PubMed: 17110040]
- Nicholls, JG. Magnes Lecture Series: Volume II. Sunderland, Massachusetts: Sinauer Associates Inc; 1987. The Search for Connections: Study of Regeneration in the Nervous System of the Leech.
- Poon CS, Young DL. Nonassociative learning as gated neural integrator and differentiator in stimulus-response pathways. *Behav Brain Funct* 2006;2:29. [PubMed: 16893471]
- Ramón y Cajal, S. Degeneration and Regeneration of the Nervous System. New York: Hafner; 1928.
- Sahley CL, Modney BK, Boulis NM, Muller KJ. The S cell: An interneuron essential for sensitization and full dishabituation of leech shortening. *J Neurosci* 1994;14:6715–6721. [PubMed: 7965072]
- Salter MW. Cellular neuroplasticity mechanisms mediating pain persistence. *J Orofac Pain* 2004;18:318–324. [PubMed: 15636015]
- Walters ET, Ambron RT. Long-term alterations induced by injury and by 5-HT in *Aplysia* sensory neurons: convergent pathways and common signals? *Trends Neurosci* 1995;18:137–142. [PubMed: 7754525]

Figure 1.

Schematic diagrams of the preparations. **A.** Preparation used for behavioral experiments consisted of the head and initial 11 segments. These were intact and, in response to a test stimulus applied anteriorly, exhibited reflexive shorting as measured with a tension transducer. The stronger, sensitizing stimuli were delivered to silver wires tied to the body wall of segment 10. Activity of the S cell was monitored with a suction electrode applied to the connectives posterior to ganglion 13. **B.** Ganglion 12 was exposed and a suction electrode attached to the posterior connectives for recording S-cell activity. For intracellular recording from the S-cell and from sensory neurons in ganglion 4, the skin remained attached to one side, to which the test stimulation was delivered.

Figure 2.

Intracellular recording to measure conduction along the S-cell chain. Configuration as in Fig. 1B, with intracellular recording from S-cell in ganglion 9 (S9) and stimulating suction electrode 1 on anterior connectives at the time indicated by the artifact at the start of each trace. **A.** Response to anterior connective stimulation in Sham operated animal, in which threshold for the S-cell axon was low and the impulse rapidly propagated along the chain and into the soma of S9. **B.** In axotomized (Lesion) animals, action potentials generated by the anterior suction electrode (electrode 1) propagated into S4 (data not shown) but not through the lesion, so that only with stronger stimulation producing EPSPs in S9 was it possible to elicit action potentials. **C.** In 1 of 13 animals in the Early Regeneration group, from 4 to 5 weeks, there was intermittent through-conduction; at higher threshold, EPSPs activated action potentials, but with a greater delay. Conversely, posterior connective stimulation could directly activate action potentials in S4 (not shown), confirming regeneration of the S9 axon. **D.** Preparation in which the S9 axon had regenerated. Resting potentials were 42–44 mV in A, C, and D and 54 mV in B. Different artifact amplitudes do not indicate different stimulus strengths.

Figure 3.

The amplitudes of shortening responses to test stimuli as a percentage of baseline contraction (indicated by a broken line at 100%), $\pm$ S.E.M. Individual responses were averaged in four blocks of 5 consecutive responses (abscissa, Blocks 1 through 4). Leeches in each experimental group experienced the sensitizing stimulus immediately following the tests of baseline responding (indicated by the downward arrows). Leeches in each control group did not receive this sensitizing stimulus, but received an identical stimulus after Block 4 (upward arrows). In Panels A–C, data are presented for the group receiving sensitizing training (filled circles) and the control group (open circles). The Mean Normalized Sensitization (MNS) is presented as filled squares in panels A–C and as various symbols in Panel D, as indicated in the inset.

Figure 4.

The amplitudes of reflexive shortening, as a percentage of baseline contraction (indicated by broken line at 100%) $\pm$ S.E.M, presented according to condition and time relative to axotomy. Thus Shams were without axotomy, Lesions were those without S-cell axon regeneration, Intermediate were those with functional S-cell axon regeneration but without capacity for sensitization of the whole-body shortening reflex, and Recovery were those with both functional S-cell axon regeneration and capacity for sensitization. The individual shortenings following sensitizing stimulation (delivered after baseline stimulation, as indicated by arrow) were grouped in 4 blocks of 5 consecutive responses (abscissa, Blocks 1 through 4).

Figure 5.

Regeneration of the S-cell axon preceded recovery of capacity for sensitization. Animals in each weekly timeframe (1-to-6 weeks following axotomy) were divided according to their behavioral and physiological characteristics—whether conduction was interrupted (LESION) or restored without recovery of sensitization (Intermediate, INTER), or was restored with recovery of capacity for sensitization (Recovery, RECOV). The number of animals in each category (above each bar) was plotted across time from axotomy (1, 2–3, 4–5, or 6 weeks). Chi-square test for independence revealed that the variables in the groups of axotomized animals were significantly associated to the time after axotomy ($p < 0.0001$). Asterisk (*) indicates significant difference from Baseline at $p < 0.05$.

Figure 6.

Excitability of the S-cell as measured by the number of action potentials in response to a test stimulus. Although whether the S-cell axon had been cut did not affect the baseline number of action potentials to a test stimulus, the operation and the time for recovery affected the response after the sensitizing stimulus. In Sham animals after a sensitizing stimulus, the number of action potentials produced by a test stimulus increased significantly, whereas in lesioned animals before regeneration (Lesion) the number dropped significantly, which was not true following regeneration. Asterisk indicates significant difference from Baseline at $p < 0.05$ (*) and $p < 0.01$ (**).

Figure 7.

Excitability of the S-cell in midbody ganglion 4 (S4) as measured by the magnitude of a 20 ms current pulse required to bring the neuron to threshold. There was no significant difference between baseline thresholds among the groups, although there were differences following the sensitizing stimulus. Whereas in Sham controls the threshold dropped significantly in some time blocks following a sensitizing stimulus, this was not true of the axotomized preparations, including the Late Regen (i.e. late regeneration) preparations.

Figure 8.

Responses of S9 to connective stimulation posterior to S12. A, *left*: In sham-operated animals, the evoked action potentials were direct and not associated with synaptic input. A, *middle* and *right*: Following axotomy of S9, an excitatory postsynaptic potential (EPSP) (*middle*) and an EPSP followed by an inhibitory postsynaptic potential (IPSP) (*right*) were evoked by stimulation of the posterior connective. B, *left*: Below threshold for direct activation of the S-cell axon, increasing stimulation sometimes first recruited an EPSP, followed by additional recruitment of an IPSP. B, *right*: Depolarization of the cell membrane from the resting potential level of -50 mV enhanced the IPSP, while hyperpolarization decreased and abolished it, consistent with an IPSP generated by a conductance increase.

Figure 9.

Distribution of sub-threshold synaptic responses in S9 to the stimulation of posterior connective, as in Fig. 8, according to the degree of repair. Posterior connective stimulation did not trigger synaptic activity (open bars), or caused only EPSPs (gray bars), or caused EPSPs followed by IPSPs (black bars) for each experimental group. These data indicated that although the biphasic EPSP/IPSPs were seen in some unoperated preparations, S-cell axotomy considerably increased the likelihood of its appearance in a response to posterior connective stimulation below threshold for the S-cell. χ^2 -test for independence revealed that the variables in the experimental groups were significantly associated to the incidence of subthreshold synaptic responses to posterior connective stimulation ($p < 0.0001$).

Table 1

Thresholds for activation of T- and P-sensory neurons by 20 ms depolarizing current pulses (nA).

	SHAM	Lesion	Regeneration
T-cells	1.11±0.16	1.08±0.16	1.11±0.11
P-cells	1.13±0.18	1.08±0.15	1.12±0.15

Table 2

Number of action potentials recorded in T- and P- sensory neurons in response to test stimulation of skin.

	SHAM	Lesion	Regeneration
T-cells	0.65±0.43	1.69±0.34	1.45±0.27
P-cells	1.04±0.42	1.40±0.39	1.84±0.39