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Effects of Nerve Injury and Segmental Regeneration on the Cellular Correlates of Neural Morphallaxis

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ABSTRACT Functional recovery of neural networks after injury requires a series of signaling events similar to the embryonic processes that governed initial network construction. Neural morphallaxis, a form of nervous system regeneration, involves reorganization of adult neural connectivity patterns. Neural morphallaxis in the worm, *Lumbriculus variegatus*, occurs during asexual reproduction and segmental regeneration, as body fragments acquire new positional identities along the anterior–posterior axis. Ectopic head (EH) formation, induced by ventral nerve cord lesion, generated morphallactic plasticity including the reorganization of interneuronal sensory fields and the induction of a molecular marker of neural morphallaxis. Morphallactic changes occurred only in segments posterior to an EH. Neither EH formation, nor neural morphallaxis was observed after dorsal body lesions, indicating a role for nerve cord injury in morphallaxis induction. Furthermore, a hierarchical system of neurobehavioral control was observed, where anterior heads were dominant and an EH controlled body movements only in the absence of the anterior head. Both suppression of segmental regeneration and blockade of asexual fission, after treatment with boric acid, disrupted the maintenance of neural morphallaxis, but did not block its induction. Therefore, segmental regeneration (i.e., epimorphosis) may not be required for the induction of morphallactic remodeling of neural networks. However, on-going epimorphosis appears necessary for the long-term consolidation of cellular and molecular mechanisms underlying the morphallaxis of neural circuitry. *J. Exp. Zool. (Mol. Dev. Evol.) 310B:520–533, 2008.*

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The aquatic worm, *Lumbriculus variegatus*, replaces lost body parts after amputation by activating two distinct developmental processes: epimorphosis and morphallaxis (Drewes and Fourtner, '90; Lesiuk and Drewes, 2001a; Martinez et al., 2005). Epimorphosis involves stem cell differentiation and blastema formation during the compensatory replacement of lost body segments. Morphallaxis, the reorganization of original (intact) structures without the recruitment of cell proliferation (Morgan, '01; Gilbert, 2006; Sanchez-Alvarado and Tsonis, 2006), remodels existing neural networks. Neural morphallaxis involves the transformation of the adult nervous system as regenerating fragments acquire new anterior–posterior neurobehavioral identities (Drewes and Fourtner, '90; Lesiuk and Drewes, 2001a; Martinez et al., 2005, 2006). This rare form of neural plasticity is highly adaptive, because segmental regeneration in *Lumbriculus* is asymmetric; that is, body fragments stereotypically regenerate only eight new head segments on anterior ends and tails of variable length on posterior ends (Drewes and Fourtner, '90; Lesiuk and Drewes, 2001a; Martinez et al., 2005). Thus, as new head and tail buds develop by epimorphosis, original segments generally acquire a more anterior body position. This shift in axial position is accompanied by morphallactic transformation of sensory, interneuronal, and motor pathways (Drewes and Fourtner, '90; Martinez et al., 2005).

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Neural morphallaxis is also activated earlier to body fragmentation by architomic fission during asexual reproduction (Martinez et al., 2005, 2006). Although the use of the terms epimorphosis and morphallaxis has recently come into question (Agata et al., 2007), in lumbriculid worms these terms appropriately define distinctive regenerative processes that underlie the replacement of lost structures and the remodeling of others. The replacement of lost segments in *Lumbriculus* occurs as a process of epimorphosis, which involves the formation of a wound blastema that further differentiates into new head and tail body parts (Morgullis, '07; Berrill, '52; Drewes and Fourtner, '91). As amputated fragments always regenerate a head of 7–8 segments in length, the original fragment segments undergo a morphallactic regeneration, which involves anatomical, physiological, and behavioral reorganization of those segments (Drewes and Fourtner, '91; Martinez et al., 2005, 2006). Specifically, worm fragments demonstrate changes in nervous system structure; in rapid-escape reflexes; and in regeneration-specific protein expression (Drewes and Fourtner, '91; Martinez et al., 2005, 2006).

Although *L. variegatus* possesses a remarkable capacity for regeneration, this ability varies within the annelid phyla, as it does across the metazoan phylogeny (Berrill, '52; Sanchez-Alvarado, 2000; Brockes et al., 2001; Sanchez-Alvarado and Tsonis, 2006). Leeches, for example, are completely incapable of regenerating lost segments, whereas other annelids regenerate entire new individuals from small body fragments (Morgulis, '07; Okada, '29; Berrill, '52). Annelid worms are particularly limited in their ability to regenerate anterior body parts, whereas posterior segment regeneration is common and perhaps an ancestral character of the phylum (Bely, 2006). Lumbriculid worms have long been known as a favorable oligochaete for experimental studies of regeneration on account of their large body size, their high capacity for regeneration, their low mortality, and their ease of laboratory manipulation. Additionally, segmental regeneration in these worms has inherent qualitative and quantitative differences along the animals' anterior–posterior axis (Hyman, '16), thus linking regeneration mechanisms with regulators of body pattern.

Studies of regeneration in *L. variegatus* have allowed the investigation of the unique contributions of epimorphosis and morphallaxis to the neural plasticity that follows injury or reproductive fission. We have utilized changes in rapid escape reflex behavior (Drewes and Fourtner, '91; Martinez et al., 2006) and a molecular marker of morphallaxis (Martinez et al., 2005) to examine the contributions of nerve injury and segmental regeneration in the remodeling of the original body fragment. A neural glycoepitope, which is labeled by a monoclonal antibody, Lan 3-2 (Martinez et al., 2005), allowed the monitoring of several proteins whose expression is enriched during morphallaxis. These morphallaxis-associated proteins include a 66 kDa protein, possessing a mannose-rich glycoepitope whose upregulation corresponds in time with neural morphallactic changes in anatomical and physiological aspects of escape pathways. Lan 3-2 labels a neural glyco-domain in the leech nervous system and has been shown to be differentially upregulated during axonal development in the leech nervous system (Zipser and McKay, '81; Zipser et al., '89; Song and Zipser, '95; Huang et al., '97; Tai and Zipser, '98, '99, 2002; Jie et al., '99). In *L. variegatus*, Lan 3-2 also labels neural structures, including axonal tracts and giant fiber pathways of the central nervous system (Martinez et al., 2005). The inductive events of neural morphallaxis, our data here suggest, include damage to the central nervous system, regardless of whether or not that induction was triggered by stimuli associated with body injury or the activation of developmental programs for reproductive fission. Moreover, ensuing epimorphosis was necessary for the long-term consolidation of the anatomical and physiological correlates of neural morphallactic plasticity.

MATERIALS AND METHODS

Animals and maintenance

Worms (*L. variegatus*) were purchased from Flinn Scientific, Inc. (Batavia, IL). They were housed in aerated spring water (Ozarka; Moffit Spring, TX), at a constant temperature of 16°C (± 1) in the dark. Worm cultures contained brown paper towel clippings for substrate and were fed spirulina powder and Tetramin staple flakes twice weekly.

To obtain experimental body fragments from specific body regions (anterior or posterior), worms were briefly anesthetized in 0.25 mM nicotine (Sigma; St. Louis, MO) in spring water. Segmental amputation was made at intersegmental boundaries with microdissecting scissors. Body fragments consisted of approximately 30 segments from the anterior third or posterior third of the worm.

Asexual fission was induced by exposure to an environmental shift involving transfer of worms from standard culture conditions to those maintained at room temperature (22°C) that lacked paper substrate and aeration for 3–4 days. Worms were then returned to normal culture at 16°C. Ninety percent of worms exposed to this temperature shift undergo asexual reproduction within 3 weeks (Martinez et al., 2006). In experiments investigating the ability of amputation to suppress asexual reproduction, worms of 150 segments in length were cut into two body pieces, at segment 100, 2 days after temperature shift.

Ectopic head (EH) formation

Worms of 150 segments in length were paralyzed in 0.25 mM nicotine, immobilized to a sylgard block, and partially transected on either the dorsal or ventral body surface. Specifically, 1–5 segments of body wall, together with either dorsal blood vessel (DBV) or ventral nerve cord (VNC) were surgically ablated. Four segmental locations of wounding were studied: segments 25–30, 50–55, 75–80, and 100–105. Recovering animals were kept individually isolated in plastic ice cube trays filled with spring water at room temperature.

Boric acid treatment

Whole animals or body fragments were immersed in spring water containing boric acid (99.9% purity; 100 pM–50 mM) for 2 weeks. Animal survival and segmental regeneration were monitored daily. Newly regenerated head and tail buds were identified by the presence of defined characteristics such as segmental boundaries and vascular organization. New segments were counted and measurements of fragment weight, sensory fields, and giant fiber conduction velocity were calculated. At the conclusion of exposure and testing periods, animals were prepared for protein extraction.

Giant fiber sensory field mapping

Impulse conduction along giant nerve fibers was studied using noninvasive electrophysiological recordings (O'Gara et al., '82; Drewes and Fourtner, '90). Touch stimuli were delivered by a handheld plastic probe. Medial giant fiber (MGF) and lateral giant fiber (LGF) action potential waveforms were distinguished based on previously reported spike characteristics (Drewes and Fourtner, '89,'90; Rogge and Drewes, '93). Extracellular voltage recordings were obtained using a printed-circuit-board grid of electrodes and electrical signals were preamplified using a pair of differential recording amplifiers (100 × gain, AC-coupled inputs). These spike recordings were digitized with a Powerlab A–D conversion system (ADInstruments, Inc.; Colorado Springs, CO) and were analyzed on a G4 Macintosh computer (Apple, Inc., Cupertino, CA) using the Powerlab Chart software. Noninvasive recordings were used to map giant fiber (touch-activated) sensory fields. Segments of specific identity (e.g., segment number 50) were marked with a spot of water-insoluble ink from a fine tip pen (Sharpie). Individual segments were then touched with a probe and giant fiber electrophysiological responses and overt segmental shortening were observed.

SDS-page and Western blotting

Experimental fragments were cultured for 3 weeks post-amputation. Prior to homogenization, newly formed head and tail pieces where excised and discarded to remove factors specific to epimorphic tissues (i.e., forming head and tail buds). Fragments were removed from the anterior and posterior regions of asexually reproducing worms at 2 weeks post-environmental shift. During EH formation experiments, fragments were isolated from segments anterior to and posterior to the wound in both dorsal and ventral lesioned worms. In all experimental groups, worm fragments were then homogenized in osmotic lysis buffer (10 mM Tris, pH 7.4 and 0.3% SDS) supplemented with a cocktail of inhibitors (0.2 mM AEBSF, 1 mg/mL leupeptin, 0.36 mg/mL E-64, 5.6 mg/mL benzamide, 50 μg/mL RNase, 100 ìg/mL DNase in a solution of 5 mM $MgCl_2$, 500 mM EDTA, and 10 mM Tris-Cl, pH 7.0). All steps of protein sample preparation were completed on ice. SDS-PAGE was performed according to standard procedures (Laemmli, '70). Electroblot transfer was performed as in Towbin et al. ('79) using the Hoeffer system (Amersham; Piscataway, NJ) to 0.2 μm nitrocellulose (Bio-Rad, Hercules, CA). Blots were incubated with diluted antibody (Lan 3-2, 1:10, J.Jøhansen; Anti-α-Tubulin, 1:1,000, Sigma, St. Louis, MO) and visualized using anti-mouse, alkaline phosphatase-conjugated secondary antibody (1:300; Vector; Burlingame, CA). The signal was then developed with BCIP/NBT (tablets; Sigma). Stained gels or blots were digitized using a Nikon image capturing system and were analyzed using NIH Image (1.37v) densitometry analysis.

Statistics

Two-tailed student's *t*-tests (Microsoft Excel) or ANOVA (Statistica, Inc., Tulsa, OK) were used for statistical analysis. Data are presented as mean plus or minus standard deviation (SD) or standard error of the mean (SEM) as indicated. A two-sample test of proportions was used for statistical analysis of the percentages of EHs formed where $P = 0.001$. Statistical significance was $P < 0.05$.

RESULTS

Partial ablation of the VNC in *L. variegatus* results in the formation of a secondary head located in an ectopic segmental position (Fig. 1a; Lesiuk and Drewes, 2001b); however, whether specific segmental damage involving nerve cord lesion is required for EH formation remains unknown. To address the requirement for nerve cord injury in EH formation, a population of worms had five segments of dorsal body wall excised, along with the underlying DBV and gut tissue (dorsal lesions lacking nerve cord damage, DL; Fig. 1b). A parallel population of worms was given ventral ablations including nerve cord damage (ventral lesions, VL; Fig. 1b). All worms with dorsal lesions initiated wound healing at the ablation site within 24 hr of injury and no EHs were produced after 3 weeks of regeneration ($n = 25$, DL-WH animals). Worms with ventral lesions and VNC excision (at segment 100 in animals of 150 total segments), either formed EHs (28.6%; $n = 35$, VL-EH animals) or produced wound healing of the damaged body segments (71.4%; $n = 35$, VL-WH animals). Nerve cord excision studies were designed to control the amount of damage to the ventral body wall and VNC. However, it is unclear if slight differences in body damage influenced the number of EHs produced after nerve injury. Nonetheless, dorsal body wall excisions resulted in no EH formation. Despite differences in the number of EHs produced, the gross structure of the EH, although occasionally containing fewer segments, was consistent with normal head buds regenerated at anterior segments. EHs consisted of a prostomial segment and 5–8 body segments, which projected ventrally at a 90° angle to the longitudinal axis of the body (Fig. 1a). Thus, damage to, and wound healing of, dorsal body tissues (including body musculature, vasculature, and intestinal tract) were not sufficient to induce EH formation, at least in posterior segments where these lesions were produced.

Interestingly, after amputation of the original eight anterior (head) segments in all VL-EH animals tested ($n = 10$), EHs acquired the ability to regulate head-specific probing and feeding behaviors, forward crawling, and head withdrawal reflexes regardless of their axial position. The capacity of these EHs to direct body movements was then lost after regeneration of new primary head bud at the anterior position and this anterior head gained neurobehavioral control. These observations demonstrate that ectopically positioned heads are fully functional body parts. Furthermore, they suggest that although an EH becomes functionally connected at multiple system levels and regulates animal behavior, a hierarchy of behavioral control exists between potential head positions along the axial body plan, with anteriorly positioned heads being dominant.

To examine the extent to which VNC excision was necessary for EH formation, we produced ablations with varying numbers of body segments with VNC lesion. Experimental populations were created that consisted of animals with one, three, or five segments of ventral body/VNC ablation (at segment 100 in animals of 150 total segments). Each group was capable of forming EHs. One-segment VNC ablations resulted in the fewest EHs formed (7%; $n = 27$); where as, excisions of two and three segments of VNC resulted in 26% ($n = 27$), and 40% of animals ($n = 37$) with EH formation, respectively. VNC transections (simple scissor cuts; $n = 20$) did not induce EH formation. Thus, nerve cord ablation, of as little as one segment of VNC, was sufficient to induce EH formation. However, higher efficacy of EH induction occurred with significantly greater central nervous system damage. These data suggest that central nervous system damage was necessary for the subsequent generation of an secondary head, even though some forms of nerve cord damage, especially simple transection (VNC cut), were not sufficient for EH formation. Interestingly, only once (out of hundreds of VNC ablations) was the formation of an ectopic tail observed. The circumstances responsible for this rare regenerative event remain unknown.

Neural morphallaxis is detected after EH formation

Neural morphallactic reorganization of giant fiber anatomy and physiology occurs in segments posterior to newly formed EHs (Lesiuk and Drewes, 2001b). That is, earlier to lesion and EH

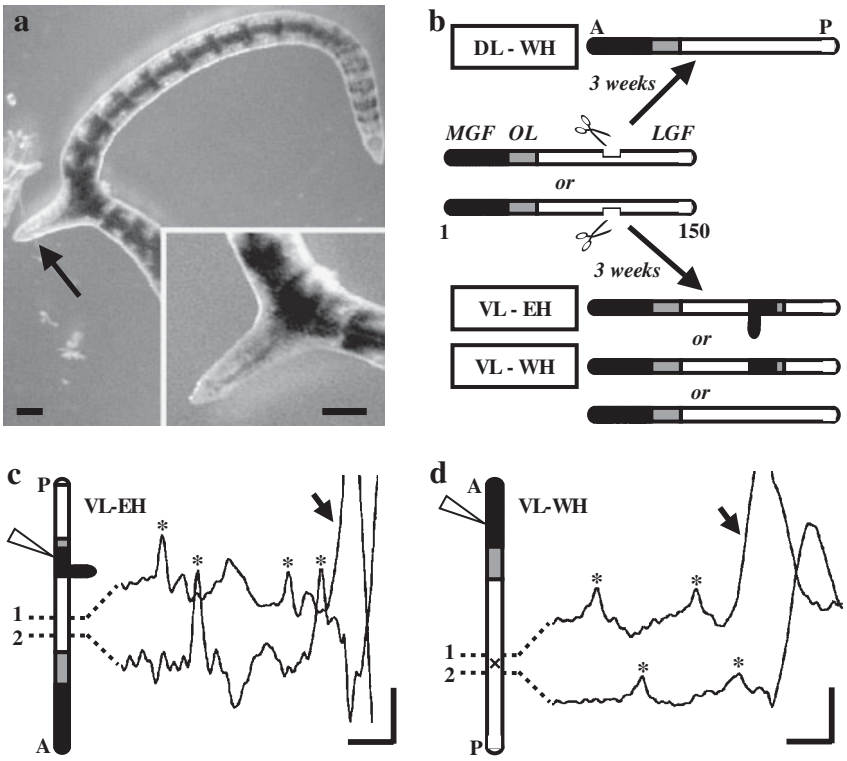

Fig. 1. Ectopic heads generated by nerve cord lesion. (**a**) Secondary heads were regenerated in ectopic positions along the body axis after removal of five segments of ventral body wall and the associated nerve cord. The normal head is located in the upper right of the image. Ectopic heads (arrow) generally contained 5–8 body segments including a prostomium. The inset presents a higher magnification of the ectopic head. Scale bars = 2 mm. (**b**) Ectopic heads in the first series of experiments were generated in worms of approximately 150 segments. Lesions were made on either the dorsal or ventral surface of posterior regions (approximately segment 100 ± 5), at segments that possessed only lateral giant fiber sensory fields (*LGF sf*, white shading). After 3 weeks of regeneration, dorsal and ventral ablations resulted in the formation of three populations of animals: dorsal lesioned animals that had undergone wound healing (DL-WH); ventral lesioned animals that had formed ectopic heads (VL-EH); and ventral lesioned animals that had undergone wound healing (VL-WH). Changes in giant fiber sensory fields were detected only in ventral lesioned animals. Medial giant fiber sensory fields (*MGF*, black shading) emerged in segments posterior to the lesion site, irrespective of ectopic head formation. Dorsal lesioned animals never produced ectopic heads or exhibited changes in sensory fields. Areas of sensory field overlap (*OL*, shaded gray) indicate regions where touch elicited both MGF and LGF spikes. A indicates anterior and P indicates posterior. (**c**) Medial giant fiber sensory fields were detected using noninvasive electrophysiological recordings in ventral lesioned animals with ectopic heads (VL-EH). After tactile stimulation (white arrow head) to segments just posterior to a newly formed ectopic head, MGF spikes (indicated by asterisks), which propagated along the length of the worm, were detected at two recording sites along the animal's body (numbers 1 and 2). Characteristic compound muscle potentials (black arrow) were also recorded. These large MGF spike-activated muscle potentials were associated with segmental shortening and withdrawal responses in both the original and ectopic heads. Giant fiber spikes are presented propagating toward the anterior end of the animal (the original head), as indicated by the MGF occurring first at recording site 1. The distance between electrode recording sites was 5 mm. Scale bars = 100 μV (vertical bar) and 0.5 ms (horizontal bar). (**d**) Medial giant fiber spikes were conducted across nerve cord transection sites in a ventral lesioned animal after 3 weeks of wound healing (VL-WH). Tactile stimulation (white arrow head) of the head region of the animal resulted in through-conducting MGF spikes (asterisks), indicating that the ventral nerve cord had regenerated across the 5-segment lesion zone (designated by the X between recording sites 1 and 2). Activation of a characteristic MGF-evoked muscle response was also detected (black arrow) and was associated with segmental shortening and a head withdrawal response. Giant fiber spikes are presented propagating toward the posterior end of the animal, as indicated by the MGF occurring first at recording site 1. The distance between electrode recording sites was 5 mm. Scale bars = 100 μV (vertical bar) and 0.5 ms (horizontal bar). A indicates anterior and P indicates posterior.

formation, posterior segments possess only LGF sensory fields and activate only tail shortening behavior (Lesiuk and Drewes, 2001b; Martinez et al., 2005). However, after ventral lesion, MGF spikes were activated in these segments (Fig. 1c). This morphallactic reorganization was present in 100% of ventral lesion animals with EHs (VL-EH) and was comparable to changes that occur in body fragments during segmental regeneration (Drewes and Fourtner, '90) and during asexual

reproduction (Martinez et al., 2006). In contrast, tactile stimulation of segments just posterior to the site of injury in dorsal lesion worms that had undergone wound healing (DL-WH) elicited no MGF activity (Fig. 1b). Thus, maps of MGF and LGF touch sensory fields in segments with dorsal injury were not different than those of intact worm segments ($n = 12$). Ventral lesion worms that sustained VNC damage, but did not form EHs (VL-WH), developed variable changes in touch sensory fields just posterior to the site of wound healing at 3 weeks after injury. Tactile stimulation to segments 101–110 resulted in MGF responses in 24% of the preparations (Fig. 1b; VL-WH, $n = 25$).

Normal LGF sensory fields were present in all other animals (76%) lacking EHs (Fig. 1b). In these ventral lesion animals (VL-WH), wound healing included compensatory regeneration of the VNC, as indicated by reestablishment of through-conducting giant fiber pathways within 3 weeks of ablation (Fig. 1d). Thus, nerve cord injury induced neural morphallaxis, in some cases (24% of VL-WH animals), even in the absence of EH formation. In all cases of morphallaxis in the absence of EH formation, changes in GF sensory fields were transient and were not detected 4 weeks after injury ($n = 6$).

We have identified molecular markers of neural morphallaxis using a monoclonal antibody, Lan 3-2 that labels proteins possessing a mannosidic epitope that are upregulated during neural morphallaxis (Zipser and McKay, '81; Martinez et al., 2005). In *Lumbriculus*, Lan 3–2 immunostaining is localized to periaxonal regions of the giant fiber pathways; with diffuse staining detected near the extracellular surface of the giant axons and their surrounding glial sheaths. Moreover, Lan 3-2 staining is localized primarily to lateral giant axons within the VNC and distinct axons within the segmental nerves (Martinez et al., 2005). The expression of Lan 3-2 epitope-bearing proteins was examined in ventral lesion and dorsal lesion worms. Western blots of protein extracts from dorsal lesion animals (DL-WH; Fig. 2) were not different from those typically obtained from intact worms (Martinez et al., 2005). The Lan 3-2 positive proteins in these DL-WH animals included a group of high-molecular weight proteins (between 210 and 130 kDa; Fig. 2). In VL-EH animals with VNC ablations and EH formation, there was significantly greater density of these high-molecular weight proteins. Moreover, there was a marked induction of a 66 kDa protein

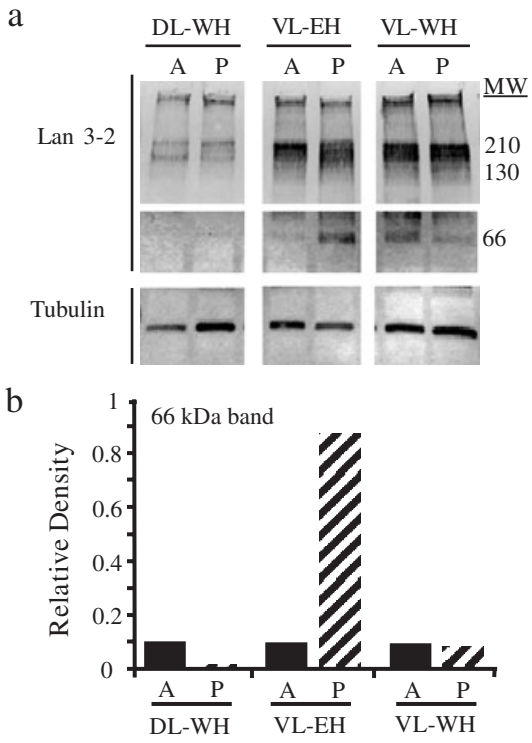

Fig. 2. Lan 3-2 epitope upregulation after nerve cord lesion. (**a**) Lan 3-2 epitope expression was upregulated in segments of ventrally lesioned animals (VL) that generated ectopic heads (EH) or wound healing responses (WH). Western blot analysis of protein extracts from dorsally lesioned animals (DL-WH) revealed protein bands near 210 and 130 kDa and these high-molecular weight protein bands were enriched in VL animals. A 66 kDa Lan 3-2 positive protein, a putative molecular marker of neural morphallaxis, was not highly expressed in DL-WH animals, but was enriched in ventrally lesioned animals in segments posterior to the forming ectopic head (VL-EH; column P). The 66 kDa band was detectable with the Lan 3-2 antibody both anterior and posterior to the site of wound healing in VL-WH animals (columns A and P). Tubulin antibody was utilized as a loading control. (**b**) Quantification of the 66 kDa morphallaxis marker protein in Western blots was performed with densitometry and was expressed as the ratio of 66 kDa band relative to the density of the tubulin loading control band. Densitometry demonstrated that expression of this protein was 7–8-fold greater in segments posterior (P) to the ectopic head of VL-EH animals than in any other protein extract groups. Solid bars indicate densitometry data from extracts of segments anterior (A) to lesion sites and striped bars indicate data from segments posterior (P) to the lesion sites.

(or enhanced glycosylation of the protein) posterior to the newly formed EH (Fig. 2). This 66 kDa protein, which is upregulated during neural morphallaxis in regenerating fragments (Martinez et al., 2005) and at fission zones earlier to asexual reproduction (Martinez et al., 2006), was not detectable in dorsal lesion animals (Fig. 2). This morphallaxis marker was mildly enriched

in extracts from segments both anterior and posterior to the ablation site in VL-WH animals that lacked EHs (Fig. 2). Thus, upregulation of the 66 kDa morphallaxis protein marker correlated with the emergence of MGF sensory fields only in segments posterior to the newly formed EHs. Moreover, all worms that incurred nerve cord damage, whether forming EH or not, exhibited strong Lan 3-2 positive staining of higher-molecular weight proteins.

Populations of worms were generated with VNC ablations at varying segmental positions along the anterior–posterior body axis (Fig. 3). Four segmental positions were preselected as lesion sites (approximately centered at segment 25, 50, 75, and 100) and five segments of VNC were ablated in all cases. Identical to previous experiments for lesions located at segment 100, all worms injured on the dorsal body surface failed to produce EHs, irrespective of the lesion position ($n = 121$; Fig. 3). In contrast, EHs were formed by some worms with VNC ablation at every segmental level tested (Fig. 3). A significantly higher percentage of EHs ($P < 0.005$) was produced at segment 50, an axial position previously defined as the zone of MGF/LGF sensory field overlap and the primary site of asexual fission plane formation (in animals 150 segments in length; Martinez et al., 2005, 2006). Thus, this region of the animal's body axis is particularly plastic in regards to neurobehavior, physiology, asexual reproduction, and segmental regeneration.

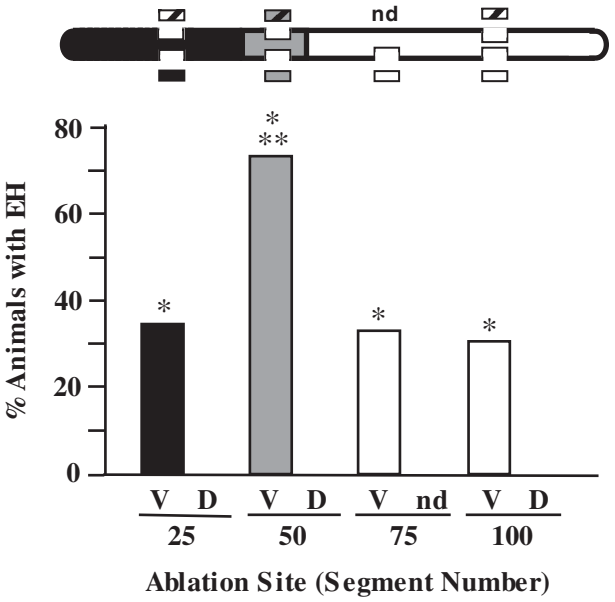

Fig. 3. Segment position-dependent formation of ectopic heads. The dorsal–ventral location and anterior–posterior segmental position of ablation sites (upper illustration) affected the percentage of ectopic heads produced in lesioned animals (lower histogram). Dorsally (D) lesioned worms did not form ectopic heads, irrespective of the segmental position of the ablation. However, ectopic heads produced in ventrally (V) lesioned worms varied depending on the segmental position of the lesion. Specifically, ectopic heads were produced in 71% of worms ($n = 23$) with ventral ablations at segment 50 (within the zone of giant fiber sensory field overlap; gray area in illustration). This percentage was significantly greater than those found at other ventral lesion sites (**, $P < 0.05$; segment 25, $n = 43$; segment 75, $n = 30$; segment 100, $n = 55$), whether within MGF (black area) or LGF sensory fields (white area of the illustration). The percentage of animals with ectopic heads at ventral lesion sites was significantly greater than corresponding dorsal lesion sites where data were collected (*, $P < 0.05$; nd indicates no data collected).

Induction of neural morphallaxis is independent of segmental regeneration and asexual fragmentation

Neural morphallaxis, including changes in giant fiber sensory fields and upregulation of protein markers, occurred not only in animals with EHs but also in some lesioned animals without EH formation, suggesting that epimorphic regeneration (in this case head regeneration) may not be necessary for induction of morphallactic events. To test whether ongoing epimorphic regeneration is required for the induction of neural morphallaxis, we measured cellular and molecular correlates of morphallaxis in regenerating fragments treated with boric acid, a chemical known to suppress regeneration of head and tail buds in this worm (Martinez et al., 2006). Regenerating fragments amputated from both anterior and posterior body regions were treated with 10 mM boric acid and were processed for protein extraction (Fig. 4a). Lan 3-2 epitope induction was not affected by boric acid treatment (Fig. 4b and c). That is, the 66 kDa morphallaxis marker protein was detected at higher levels in posterior fragments than in anterior fragments. Thus, ongoing epimorphosis (i.e., head bud formation), was not necessary for induction of a feature diagnostic of neural morphallaxis.

Lumbriculus reproduces asexually by the process of architomy. This type of asexual reproduction involves fragmentation at an intrinsically determined fission site. This predictable zone of architomy in *Lumbriculus* is segment 48 ± 10 (in worms with 150 segments), and the fragmentation event results in the consistent production of two body pieces, a head and tail zooid of similar

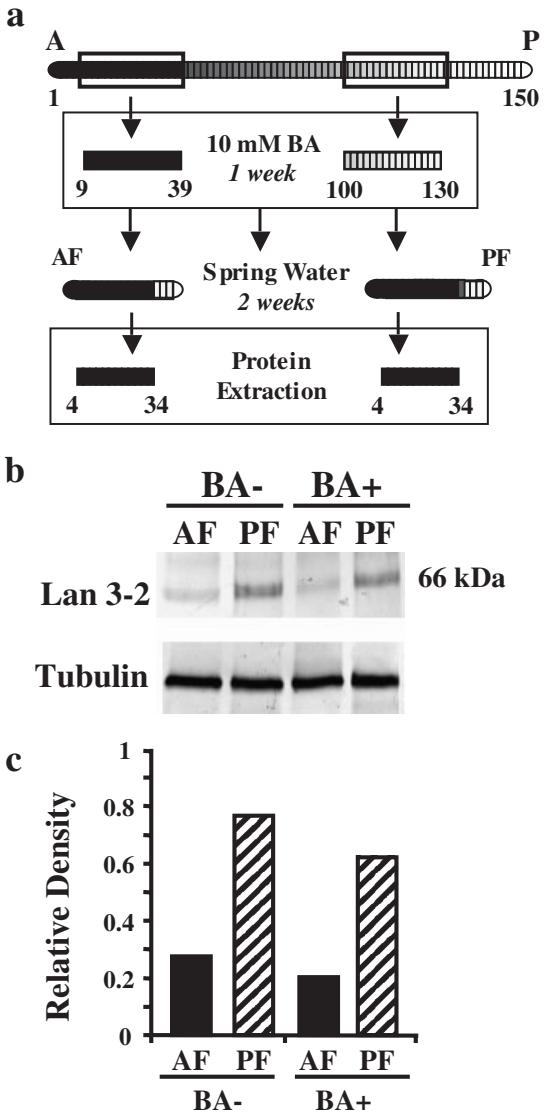

Fig. 4. Lan 3-2 epitope upregulation during diminished segmental regeneration. (**a**) Protein extracts were produced from worm fragments treated with 10 mM boric acid for 1 week. As indicated in the illustration, very few new segments formed after boric acid treatment. Boric acid treated zooids and controls incubated in only spring water for 1 week (not shown) were then cultured for an additional 2 weeks earlier to homogenization and protein extraction. Before protein analysis, any newly formed head or tail segments were removed and discarded. A and P designates anterior and posterior. AF and PF indicate anterior and posterior fragments, respectively. Segment numbers are defined below each illustrated animal. (**b**) Upregulation of the 66 kDa Lan 3-2 positive protein was not affected by boric acid treatment (BA+) as compared with Western blots of control fragments (BA-) treated only with spring water. Note that the 66 kDa protein band was enriched in posterior fragments (PF), but not in anterior fragments (AF). Tubulin expression was not affected by boric acid and was used as a gel loading control. (**c**) Quantification of the 66 kDa morphallaxis marker protein in Western blots was performed with densitometry and was expressed as the ratio of 66 kDa band relative to the density of the tubulin loading control band. Densitometry demonstrated that expression of this protein was 2–3-fold greater in posterior fragments (PF), as compared with anterior fragments (AF), regardless of whether they were treated with boric acid (BA+) or were treated with spring water (BA-).

biomass (Martinez et al., 2006). Boric acid treatment also potently blocks fission during asexual reproduction (Martinez et al., 2006). Therefore, two populations of worm cultures were induced into asexual reproduction by a shift in water temperature from 16 to 22°C (Fig. 5a). One population was treated with 10 mM boric acid (BA+) and the other was untreated (BA-). During the course of 3 weeks, control (BA-) animals formed fission planes and fragmented. Anterior

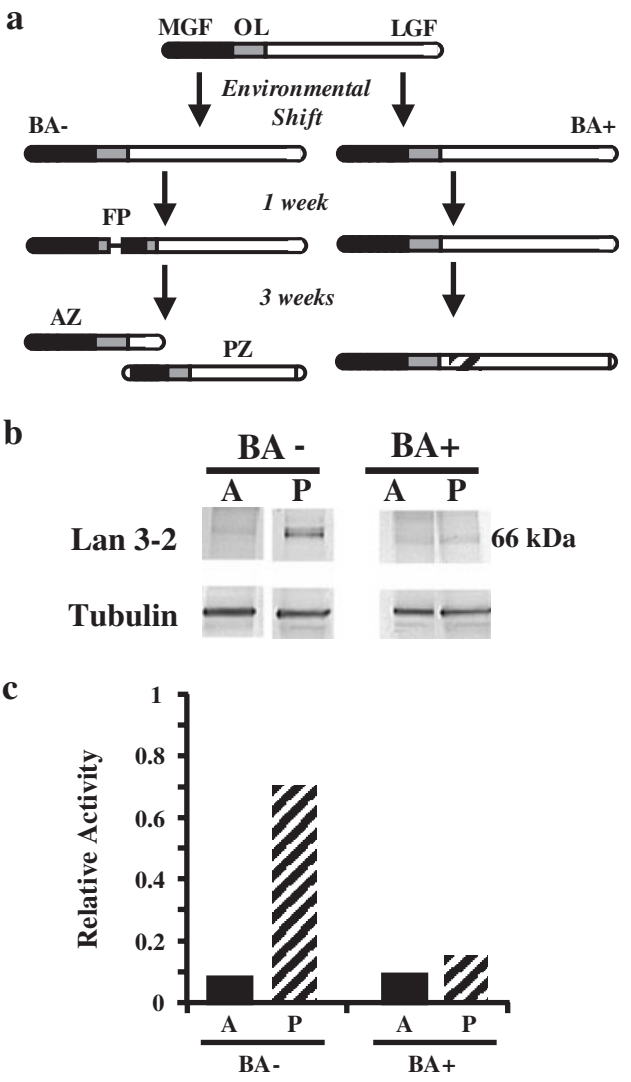

Fig. 5. Morphallaxis protein expression persists after block of asexual fission. (**a**) Animals induced into asexual fission by an environmental shift in temperature were treated with 10 mM boric acid in spring water. Control animals, not treated with boric acid (BA-), formed fission planes (PF) within the region of overlay of MGF and LGF sensory fields (gray area in the illustrations) by 1 week post-shift. The formation of fission planes was accompanied by the emergence of MGF sensory fields (black regions of the illustrations) just posterior to the fission plane. These animals fragmented into two zooids, anterior (AZ) and posterior (PZ), by 3 weeks post-shift. Animals treated with boric acid after the temperature shift (BA+) did not form fission planes and did not fragment. However, analysis of sensory fields using behavioral and electrophysiology assays revealed that weak neural morphallaxis occurred, producing changes in sensory-to-interneuronal connectivity (emergence of ectopic MGF activation; striped region of sensory field map). (**b**) Lan 3-2 epitope expression was upregulated in segments immediately posterior (P) to the fission site of control animals not treated with boric acid (BA-), but not in segments anterior (A) to the fission site. Although boric acid blocked fission plane formation in treated animals (BA+) and blocked subsequent asexual fragmentation without a significant effect on neural morphallaxis, upregulation of the 66 kDa glycoprotein was decreased, but not abolished in segments anterior (A) and posterior (P) to the predicted fission zone. Tubulin expression was also not affected by boric acid treatment and was used as a gel loading control. (**c**) Quantification of the 66 kDa morphallaxis marker protein in Western blots was performed with densitometry and was expressed as the ratio of 66 kDa band relative to the density of the tubulin loading control band. Densitometry demonstrated that expression of this protein was 6–7-fold greater in segments posterior (P) to the fission site of control animals (BA-), as compared with segments anterior to the fission site (A) in these animals. Levels of this protein were not different between anterior and posterior segments of boric acid treated animals (BA+) and both were many fold lower in their expression of this protein vs. control posterior segment levels. Solid bars indicate densitometry data from extracts of segments anterior (A) to lesion sites and striped bars indicate data from segments posterior (P) to the lesion sites.

zooids , generated by this fission, formed tail buds and posterior zooids (PZ) formed head buds. Regenerating PZs possessed MGF sensory fields that had, in large part, emerged earlier to asexual fragmentation (Fig. 5a). Fragmentation did not occur in BA-treated animals (BA+), but still exhibited the emergence of MGF sensory fields. At 1 week after the temperature shift, protein extracts produced from the body segments both anterior and posterior to the fission zone were also tested for the presence of the Lan 3-2 positive morphallaxis-associated protein (Fig. 5b and c). This protein was detected at elevated levels posterior to the fission plane in control (PZ; BA-) animals. However, in BA-treated (BA+) animals, the Lan 3-2 positive protein was only slightly elevated in segments both anterior (AS) and posterior (AP) to the fission zone (segment 50). Thus, although morphallaxis can be initiated earlier to asexual fission, its maintained expression requires body fragmentation and perhaps the epimorphosis (head or tail bud formation) that follows architomy.

Segmental regeneration suppresses asexual reproduction, but not Lan 3-2 upregulation

The formation of fission planes during asexual reproduction was aborted if the animals were also fragmented by injury at another segmental site. Asexually reproducing worms (induced by temperature shift) were transected at a segmental position (segment 100) posterior to the predicted site of fission plane formation (segment 48). Asexual fragmentation rates were then monitored

for 3 weeks (Fig. 6a). We hypothesized that the process of segmental regeneration (that of head or tail bud regeneration) in response to injury would over ride morphallactic changes in physiology, behavior, and protein expression associated with asexual reproduction as architomy was aborted. This was not what was observed. MGF spikes were activated by touch to segments posterior to the fission plane in both control animals undergoing asexual reproduction ($n = 27$; Fig. 6a) and worms amputated 1 week after the temperature shift ($n = 27$; Fig. 6a). However, in 100% of the amputated animals, asexual reproduction was aborted and well-developed fission planes were eliminated. In addition, changes in MGF sensory fields were transient and were no longer detected 3 weeks after the shift, indicating that body amputation had not only reversed the induction of architomy, but had also suppressed the consolidation of previously initiated neural morphallactic remodeling of neural circuits.

Protein extracts of both control and experimental groups were examined for induction of the morphallaxis marker protein. In asexually reproducing controls, the 66 kDa morphallaxis protein marker was detected at high levels by the Lan 3-2 antibody in segments posterior to the fission site (Fig. 6b and c). This marker protein was detected in animals exhibiting a transient form of neural morphallaxis, even though asexual fission was aborted and changes in sensory fields had been reversed.

DISCUSSION

Recovery from nerve injury involves the initiation of processes that limit the spread of damage and the induction of mechanisms necessary for the subsequent reconstruction of the compromised neural tissue. In contrast to the limited recovery of function observed in the mammalian CNS after injury, most invertebrates can readily regenerate their nervous systems and restore neural functionality with remarkable speed and fidelity (von Bernhardi and Muller, '95; Moffet, '96, 2000; Duan et al., 2005; Sanchez-Alvarado and Tsonis, 2006). The freshwater annelid worm, *L. variegatus*, is particularly adept at regenerating lost body parts and reconstructing its CNS after injury. Neural morphallaxis remodels neural circuits to compensate for changes in positional and behavioral constraints in extant segments as lost body parts are regenerated (Drewes and Fourtner, '90; Martinez et al., 2005). Additionally, neural morphallaxis occurs during asexual reproduction in anticipation of imminent body fragmentation (Martinez et al., 2006). The cellular events that induce neural morphallaxis are not well understood, however, it has been suggested that morphogenic factors, possibly emanating from newly regenerating head segments govern the induction of neural morphallaxis (Lesiuk and Drewes, 2001b). However, the suppression of epimorphic regeneration with boric acid treatment did not disrupt the induction of neural morphallaxis in either asexually reproducing or regenerating body fragments. Still, it is possible that blastema and limited bud formation provided signals sufficient for neural morphallaxis induction.

Damage to the nervous system is thought to play an important role in triggering regenerative events in both vertebrate (Dinsmore and Mescher, '98; Chang et al., 2000; Rossi et al., 2007) and invertebrate animals (Moffett, 2000; Bedi and Glanzman, 2001), including annelid worms (Drewes and Fourtner, '91; Shafer et al., '98; Bely, '99; Myohara et al., '99; Yoshida-Noro et al., 2000; Duan et al., 2005). Lesion of the CNS, but not dorsal tissues, was necessary for induction of neural morphallaxis. However, significant ablation (at least one segment) of VNC was necessary for this induction, as simple transection did not initiate sensory field changes. Induction of cellular mechanisms of plasticity, such as neural regeneration, typically requires subsequent mechanisms to maintain those changes (Makino et al., 2005). Interestingly, although damage to the VNC triggered neural morphallaxis, these changes in neural circuits were not always maintained. Animals with VNC lesions, but lacking EH formation, exhibited only transient changes in sensory fields. These results are consistent with behavioral studies that demonstrate the emergence of a transient reversal behavior in ventral lesion worms (Lesiuk and Drewes, 2001b).

Asexually reproducing animals, whose fragmentation was aborted by boric acid treatment, also exhibited transient sensory field changes. Asexual reproduction in *L.variegatus*, a type of asexual reproduction defined as architomy (Giese and Pearse, '75), involves the intrinsic lesioning of the VNC at a predictable fission site (Martinez et al., 2006). Asexual reproduction results in the formation of two clones (zooids). Not only did boric acid treated block the onset of architomy, but segmental regeneration induced by body transection aborted ongoing asexual fission, demonstrating that the early events of architomy are

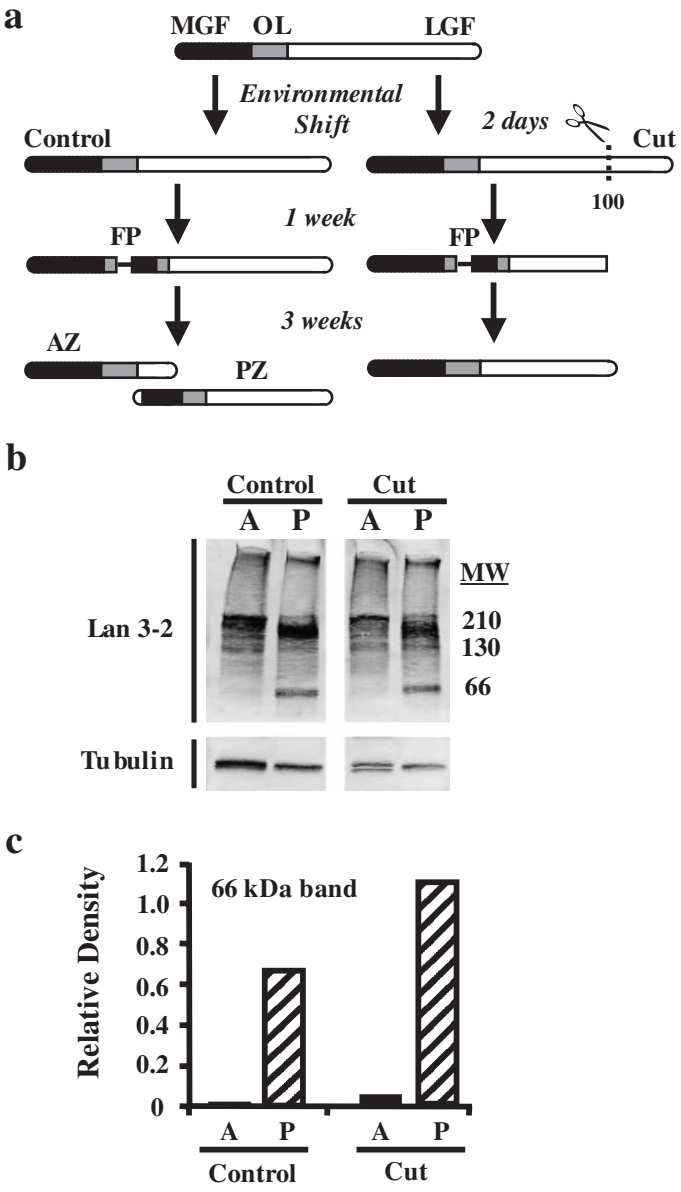

Fig. 6. Body transection blocks ongoing asexual fragmentation, not Lan 3-2 epitope upregulation. (**a**) Asexual reproduction was induced in whole animals using an environmental shift paradigm. The posterior one-third of experimental animals (last 50 segments) was removed 2 days after environmental shift by a body transection at segment number 100. A population of worms that was not amputated and continued to produce fission planes (PF) and fragment asexually into anterior (AZ) and posterior zooids (PZ). Worms that were transected did not complete asexual reproduction. Although fission planes (PF) were obvious at 1 week post-shift, they were absent by 3 weeks post-shift. Changes in giant fiber sensory fields were also reversed in posterior-cut animals by 3 weeks. Black regions of the animal illustrations indicate medial giant fiber (MGF) sensory fields. White regions indicate lateral giant fiber (LGF) sensory fields. The gray area in the illustrations indicates the region of overlap (OL) between MGF and LGF sensory fields. (**b**) Immunoblot analysis demonstrated similar Lan 3-2 epitope expression profiles in protein extracts from control (asexually reproducing) and cut (aborted asexual reproduction) animals. Protein extracts were created from segments anterior (A) to and posterior (P) to the fission site (48 ± 10). Upregulation of the 66 kDa (Lan 3-2 positive) morphallactic protein band was detected in both control and cut animals in segments posterior, but not anterior, to the site of fission plane formation. A tubulin antibody was used as a loading control. (c) Quantification of the 66 kDa morphallaxis marker protein in Western blots was performed with densitometry and was expressed as the ratio of 66 kDa band relative to the density of the tubulin loading control band. Densitometry demonstrated that expression of this protein was 6–10-fold greater in segments posterior (P) to the fission site of both animals asexually reproducing (Control) and animals whose fission has been aborted owing to body transection (Cut), as compared with segments anterior to fission sites (A) in these groups.

reversible and that initiation of epimorphosis in another body region can generate that reversal. Interestingly, the morphallactic remodeling of sensory fields was also aborted in these transected worms. One possible explanation of this aborted reproduction and morphallaxis is the onset of a distant influence triggered by body or nerve injury, such as a diffusible morphogen or transmitted neural or neuroendocrine signal. Whatever the mechanism, we propose that the maintenance of neural morphallaxis is directly influenced by epimorphic processes associated with injury or reproduction-induced fragmentation.

Taken together, our studies support the view that epimorphosis is necessary for the maintenance of neural morphallactic changes within the original worm fragment. Further, the induction of neural morphallaxis requires nerve cord damage. Thus, neural morphallaxis, like some other forms of neural plasticity, for example long-term memory formation, involves both induction and consolidation events. Learning and memory in *Aplysia* has short-term forms that encode brief behavioral changes and more persistent forms that involve consolidation mechanisms, such as structural remodeling of synaptic connections (Roberts and Glanzman, 2003). Interestingly, repair of injured neuronal axons also involves a series of events with mechanistic similarity to forms of learning (Ambron et al., '92; Dulin et al., '95; Gunstream et al., '95; Moffett, 2000; Bedi and Glanzman, 2001; Duan et al., 2005; Rossi et al., 2007). Signals generated by axonal injury trigger transcription-dependent responses, such as process outgrowth and long-lasting compensatory changes in excitability (Achee and Zoran, '96; Ambron and Walters, '96; Kumar et al., 2001; Raivich et al., 2004; Duan et al., 2005). Nerve injury-activated signals are thought to play an inductive role in nerve regeneration (Dinsmore and Mescher, '98). In the leech, nerve injury induces a rapid efflux of nitric oxide (NO), which precedes the accumulation of microglia at the site of lesion (Kumar et al., 2001). This NO-mediated microglial accumulation aids in regeneration of the injured CNS through the phagocytosis of cellular debris and the deposition of laminin to aid axonal growth (von Bernhardi and Muller, '95). Nerve injury has also been shown to induce gap junctional coupling among adult motor neurons (Chang et al., 2000). Although, in the adult mammalian nervous system, neuronal gap junctional coupling is relatively rare, it is clear that injury-induced coupling between motor neurons may mediate signaling that maintains the viability of the axotomized connection until regenerated synapses are reestablished with their targets. Similarly, it is becoming clearer that stress (or noxious stimuli) is a biologically significant factor that can disturb cognitive processes such as learning and memory (Kim and Yoon, '98; Diamond et al., '99; Kim and Diamond, 2002). It has been demonstrated in mammalian model systems that stress and stress hormones suppress induction of long-term potentiation during hippocampal-dependent memory formation (Foy et al., '87; Shors et al., '92; Kim and Yoon, '98; Mesches et al., '99, Cohen-Armon et al., 2004). These findings further strengthen the idea that molecular mechanisms underlying learning might have evolved from the cell's response to stress or injury (Walters, '94). Similarly, we have demonstrated here a role for neural injury in a rare form of regenerative plasticity called morphallaxis. Perhaps, the mechanisms of neural morphallaxis, such as synaptic plasticity underlying some forms of learning and memory, may have their evolutionary origins in anatomical and physiological responses to injury. Although the molecular events underlying neural remodeling during morphallaxis in *L. variegatus* are unknown, functional and structural modifications of giant axons and sensory-interneuronal synapses are certainly involved.

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