

Amputation-Induced Activity of Progenitor Cells Leads to Rapid Regeneration of Olfactory Tissue in Lobsters

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Received 13 August 2002; accepted 25 November 2002

ABSTRACT: Lobsters have a self-renewing olfactory system and, like many animals, continuously replace old or dying olfactory receptor neurons. In addition, lobsters are able to regenerate the peripheral olfactory system even after complete loss. The olfactory sensors in lobsters are located distally on a pair of antennules. These antennules are often damaged, but this has little impact on the lobster's sense of smell because damaged olfactory tissue is rapidly replaced. In this study, we investigated damage-induced regeneration of the olfactory system by measuring cell proliferation following controlled amputation. We show that amputation-induced regeneration occurs as a result of up-regulating the normal development of olfactory sensors. A unique feature of up-regulated development is the formation of patches of proliferating cells within the antennular epithelium. Epithelial patches were typically formed between 3 and 10 days postamputation on the amputated side. They were characterized by their:

proximal position with respect to developing clusters of olfactory receptor neurons (ORNs); tendency to form two discrete patches within the borders of each existing annulus; cell size, which was approximately twice that of mature ORNs; and location within the ventral epithelium. The development of epithelial patches was immediately followed by proliferation of clusters of ORNs and associated glial cells, and the level of this proliferation increased significantly during the premolt stage of the lobster's molt cycle. These epithelial patches may represent populations of precursor cells, because they develop in response to amputation and immediately precede development of cell clusters composed of ORNs and glia. Possible regulatory signals controlling epithelial patch development are discussed. © 2003 Wiley Periodicals, Inc. *J Neurobiol* 55: 97–114, 2003

Keywords: stem cell; neuron; glia; cell proliferation; olfaction; chemical senses; chemoreception; BrdU; Crustacea; *Panulirus argus*

INTRODUCTION

Postembryonic development of olfactory receptor neurons (ORNs) is a hallmark of many olfactory

systems (Graziadei and Monti-Graziadei, 1978; Farbman, 1992; Sandeman and Sandeman, 1996; Alvarez-Buylla and Kirn, 1997; Huard et al., 1998; Martinez-Marcos et al., 2000; Steullet et al., 2000a; Harrison et al., 2001b; Higgs and Burd, 2001). This ongoing development ensures that the size of the peripheral olfactory system is matched to body size during growth. Furthermore, it enables turnover of olfactory tissue and thus continual replacement of old or damaged neurons. Crustaceans such as lobsters offer unique models for studying postembryonic neural development because their peripheral olfactory organ—the antennule—is a continuously developing and self-renewing organ that can regenerate even after total ablation. In this article, we investigate neural proliferation and progenitor cell activity associated with regeneration of the lobster's peripheral olfactory system.

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Contract grant sponsor: National Institute on Deafness and other Communicative Disorders; contract grant number: DC00312.

Contract grant sponsor: National Science Foundation; contract grant number: IBN-0077474.

Contract grant sponsor: Georgia Research Alliance.

Contract grant sponsor: GSU Research Program Enhancement Fund.

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In mammalian olfactory systems, ORNs are produced from “immediate neuronal precursors” (INPs) that reside in the basal layer of the olfactory epithelium (Graziadei and Monti-Graziadei, 1978; Farbman, 1992). INPs are the progeny of an amplifying progenitor cell type that can be identified by its expression of MASH 1 (Gordon et al., 1995). INPs are in turn believed to be the progeny of the putative stem cell—the neuronal colony-forming cell (Mumm et al., 1996; Murray and Calof, 1999). The mammalian olfactory epithelium is self-renewing and appears capable of regeneration provided that the neuronal colony-forming cells remain intact.

Far less is known about specific cell types and ORN lineage in lobsters, but recent evidence points to common mechanisms between lobster and mammalian systems (Harrison et al., 2001a,b). Lobster ORNs are most likely produced continuously from progenitor cells in the antennular epithelium (Harrison et al., 2001b). Key differences between lobsters and mammals are that lobsters grow indefinitely throughout life and consequently the size of their peripheral olfactory system continuously increases (Fig. 1) (Hartnoll, 1981; Harrison et al., 2001b), and lobsters have the ability to regenerate peripheral olfactory tissue following injury and even after complete loss of the peripheral olfactory organ (Skinner, 1982).

The capacity to regenerate lost appendages is widespread in crustaceans (Skinner, 1982). This includes regeneration of the sense organs of these appendages (Laverack, 1988). In lobsters, the peripheral olfactory system consists of a series of repeating sensory units that are located at the distal end of the most anterior pair of appendages—the first antennae, or antennules (Fig. 1) (Laverack and Ardill, 1965; Grünert and Ache, 1988; Hallberg et al., 1992; Steullet et al., 2000b; Derby et al., 2001). Each olfactory sensory unit consists of a cuticular sensillum (aesthetasc), a cluster of about 300 ORNs, and numerous supporting cells [Fig. 1(B)] (Steullet et al., 2000b). The peripheral location of the olfactory sensory units is advantageous to lobsters in that it increases environmental sampling space (Derby and Steullet, 2001), but it also increases the chance of damage to sensors. Despite the fact that damage to the antennules is common under natural conditions (P. J. H. Harrison and C. D. Derby, personal observation), the lobster’s olfactory ability typically remains functional because there are many olfactory sensors, each of which is a repeating functional unit (Steullet et al., 2000b; Derby et al., 2001), and any damage is

rapidly repaired by the process of regeneration (Harrison et al., 2001a).

In lobsters, addition and turnover of olfactory sensory units occur continuously throughout life [Fig. 1(C,E)], and repair of minor injury to olfactory sensors appears to involve a rate-change to this normal mode of development. Turnover of ORNs has been described in detail in lobsters (Steullet et al., 2000a; Harrison et al., 2001b) and crayfish (Sandeman and Sandeman, 1996). Proliferation of new ORNs and other cells associated with developing olfactory sensory units occurs proximal to the existing population of ORNs, in a cellular region identified as the proximal proliferation zone (Harrison et al., 2001b). Development occurs continuously throughout the molt cycle, but the rate of cell proliferation is higher during the premolt stage than during intermolt, suggesting molt-dependent rate controlling mechanisms (Harrison et al., 2001b). The aesthetasc sensillar cuticle develops during the premolt stage and completes the formation of the olfactory sensory units. As new sensory units are added in the proliferation region at molting, older units are shed from a distal senescence region [Fig. 1(C)]. Under normal conditions, there is a net increase in the number of sensory units at each molt, because there is greater addition than loss (Sandeman and Sandeman, 1996; Steullet et al., 2000a). Pilot experiments for the present study suggested that amputation of olfactory sensory units is repaired by rapid up-regulation of the number of sensory units that are added and down-regulation of the number of sensory units that are lost.

In this study, we investigate antennular amputation-induced activity of progenitor cells and proliferation associated with ORN development by performing controlled antennular amputations that up-regulate ORN development. We identify putative ORN progenitors, which appear in patches of antennular epithelium shortly after antennular amputation. We show that the rate of proliferation is up-regulated following antennular amputation and that this leads to increased addition, and therefore restoration, of olfactory sensory units.

MATERIALS AND METHODS

Animals

Juvenile and young adult Caribbean spiny lobsters (*Panulirus argus*) with carapace lengths between 55 and 70 mm were used in this study. These animals were collected from the Florida Keys and shipped to Georgia State University where they were held in 800-L aquaria (20–25°C) contain-

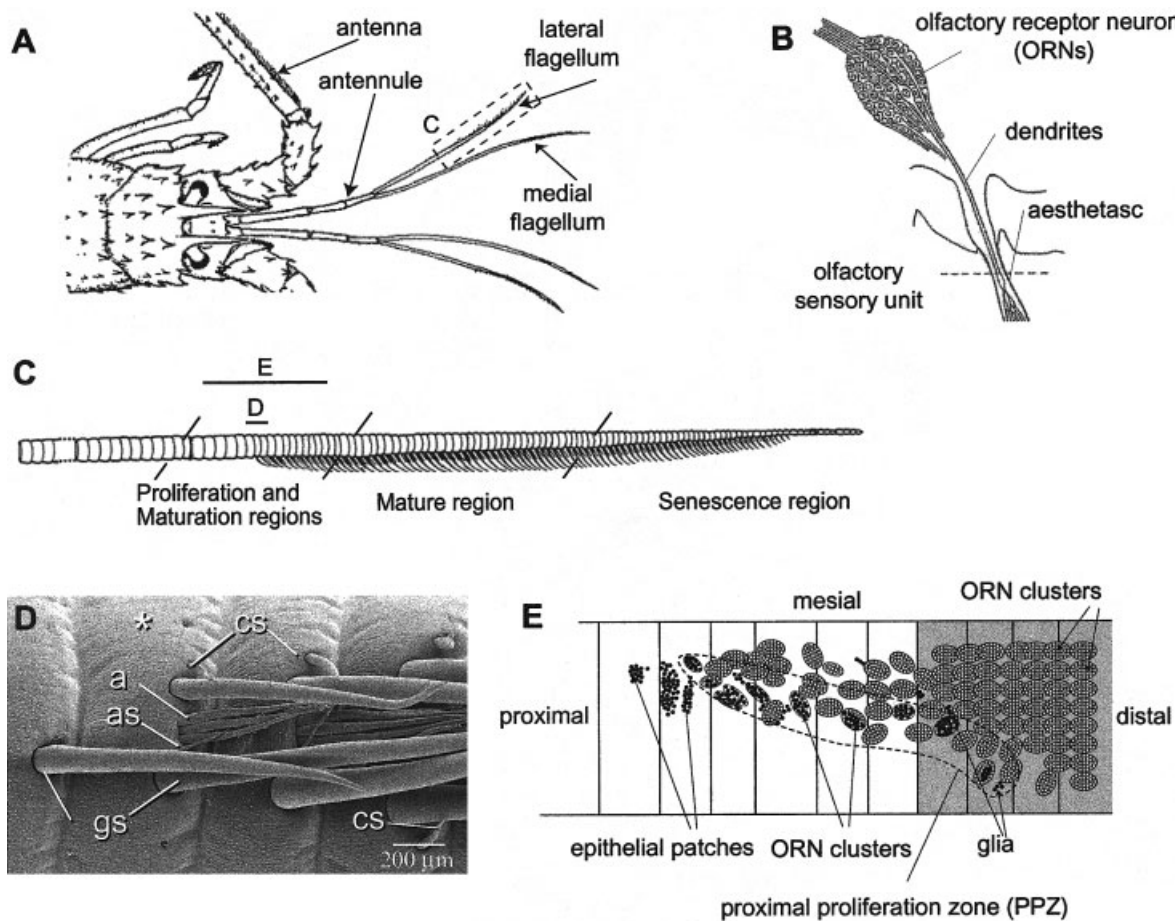


Figure 1 Postembryonic development of the spiny lobster peripheral olfactory system. Proximal is oriented to the left and distal to the right in this and all subsequent figures. (A) Olfactory sensory units are located on the lateral flagellum of each antennule. (B) Each olfactory sensory unit consists of a cluster of olfactory receptor neurons (ORNs), glial cells (not shown but part of this cluster), and the associated aesthetasc sensillum (modified from Grünert and Ache, 1988). (C,D) The lateral flagellum is composed of ringlike cuticular annuli, each housing numerous types of setae. The aesthetasc tuft is located on the ventral surface of the distal half of the lateral flagellum and is composed of aesthetasc and other setae. (C) With respect to development of olfactory sensory units, the lateral flagellum is divided into four regions. With each molt, aesthetasc sensilla are added in the proliferation and maturation regions and are lost from the senescence region. The locations of antennular regions depicted in panels (D) and (E) are indicated in (C). (D) Scanning electron micrograph showing the most proximal aesthetasc-bearing annulus (*) and neighboring annuli. Following molting, aesthetascs will be added to this annulus and those located immediately proximal (left on figure) and distal (right). a, aesthetascs; as, asymmetric seta; gs, guard setae; cs companion setae. (E) Model showing the proliferation of cells that occurs during the intermolt stage in the proximal proliferation zone of the lateral flagellum. Shaded boxes represent annuli that possess aesthetascs, and white boxes represent annuli without aesthetascs but with developing clusters [i.e., this is a similar region to that shown in (D)]. Cells proliferate (indicated by black dots) along the proximo-lateral margin of the existing ORN population (postmitotic cells indicated by gray dots). The aesthetasc sensilla associated with newly formed clusters (indicated by area within dashed line) develop late in the molt cycle (i.e., during premolt).

ing recirculating, artificial seawater (Instant Ocean™; Aquarium Systems, Mentor, OH) and fed shrimp and squid three times per week. Animals used in regeneration experiments were selected because their antennules showed no

obvious signs of damage and because examination of setal development indicated animals were in the intermolt stage (stage C) (Lyle and MacDonald, 1983). The animals were housed together in a single 800-L aquarium during the

experiments and thus were held under identical environmental conditions.

Amputation Experiments

Pilot Studies. A series of pilot studies were conducted to investigate regeneration of olfactory tissue following different degrees of injury. Restoration of the peripheral olfactory system (i.e., aesthetasc/ORN sensory units on the antennular lateral flagellum) occurred regardless of the extent of injury. Injury included the following: amputating various lengths of the lateral flagellum, by cutting off different numbers of annuli; amputating the entire lateral flagellum and medial flagellum, by cutting the antennule at the middle of the third segment; and amputating the entire antennule, by cutting the first segment near its base. The time taken to restore the antennule to its full length was dependent on the extent of the damage, but was usually complete following two to four molts. Even in extreme cases in which the first segment was cut, flagella were typically restored by the subsequent molt. Full restoration of the olfactory organ appeared complete after another two to three molts. Experiments were also conducted to monitor cell proliferation following antennular amputation. These experiments were most reliable when amputations removed ORNs but kept intact the proximal proliferation region.

Regeneration Experiments. Based on the results of the pilot studies, we focused on regeneration following amputation of most, but not all, aesthetasc-bearing annuli. In all cases, the right antennule was amputated and the left antennule remained intact to serve as a control. This type of amputation was chosen because it removed a large proportion of the olfactory system but kept intact the proximal proliferation region on each antennule, thus enabling direct comparison of proliferation rates on amputated and control antennules.

For amputations, an animal was removed from its holding aquarium and held so that the lateral and medial flagella of its right antennule lay flat on a silicone-coated Petri dish. A single cut was then made using a scalpel blade through the arthropodial membrane between two annuli on the lateral flagellum. This cut removed approximately 80% of the aesthetasc-bearing annuli. The animals were then allowed to recover for approximately 15 min or until a blood clot appeared at the tip of the amputated flagellum. Animals were then placed back in the holding aquarium. Animals used for studies of addition and loss of aesthetascs were held under these conditions until they molted. Those used to study proliferation were left for 1, 3, 7, 9, 12, 19, or 34 days. Proliferation experiments were performed on three separate occasions using animals collected at different times, and the results were pooled.

Quantification of the Addition and Loss of Aesthetasc-Bearing Annuli

To determine numbers of aesthetascs added and lost at molt, we used a method that was simplified from one used pre-

viously by Steullet et al. (2000a). Immediately following a molt, postmolt antennules were removed from an animal, and the corresponding premolt antennules were collected from the "molt shell" (exuvium). Both were fixed in 4% paraformaldehyde in 0.2 M phosphate buffer and stored at 4°C prior to quantification. To quantify addition and loss, corresponding annuli on the pre- and postmolt antennules were matched as described elsewhere (Sandeman and Sandeman, 1996; Steullet et al., 2000a), and the two antennules were aligned accordingly. Changes in numbers of aesthetasc-bearing annuli between the pre- and postmolt antennules were then determined by calculating the difference between antennules using a Zeiss stereomicroscope (Zeiss, Jena, Germany).

BrdU Immunocytochemistry

Proliferation was monitored using 5-bromo-2'-deoxyuridine (BrdU; Sigma, St. Louis, MO). BrdU was injected into the hemolymph of experimental animals (5 mg BrdU per 100 g body weight). BrdU was dissolved in *P. argus* saline, which was composed of (in mM): 458 NaCl, 13.4 KCl, 13.6 CaCl₂, 9.8 MgCl₂, 14.1 Na₂SO₄, 3 HEPES, 1.9 glucose, 1.2 NaOH (pH adjusted to 7.4). The procedure used to study BrdU labeling in spiny lobster antennules is described in detail elsewhere (Harrison et al., 2001b). Briefly, antennules were removed from the animals, fixed in Bodian #2 fixative (70% ethanol, 5% formalin, and 5% glacial acetic acid, v/v in H₂O), rehydrated, rinsed in 0.2 M phosphate buffer (3 × 10 min), and cut along the horizontal midline to obtain ventral and dorsal hemisections. Dorsal hemisections were discarded and ventral hemisections (containing olfactory sensory units) were rinsed in buffer (3 × 20 min), incubated in 2 N HCl for 30 min, rinsed in buffer (4 × 15 min), and incubated for 4 h in a blocking solution consisting of 5% normal goat serum, 1% bovine serum albumin (Sigma), 0.1% sodium azide, and 0.3% Triton X-100 in buffer. Hemisections were then incubated overnight in an undiluted droplet of anti-bromodeoxyuridine antibody (mouse monoclonal antibody + nuclease: Amersham, Arlington Heights, IL), rinsed in buffer (4 × 30 min), and incubated for 4 h in goat antimouse CY3-labeled secondary antibody (Amersham) diluted 1:1000 in buffer. To visualize cells, hemisections were pinned to silicone-coated slides and mounted in buffer containing 0.1% sodium azide. In some cases, tissue was dehydrated through a graded series of ethanol, cleared in methyl salicylate, and mounted in low viscosity Cytoseal 60 (Stephens Scientific, Kalamazoo, MI). Antibody specificity controls were not routinely performed as part of this series of experiments but have been performed and described by us elsewhere (Harrison et al., 2001b).

Confocal Microscopy, BrdU Quantification, and Statistical Analysis

Tissue was viewed and photographed on a Zeiss confocal microscope, using LSM 510 software. For all figures, proximal was oriented to the left and distal to the right. BrdU-

positive cells (BrdU⁺) were reliably identified in the majority of preparations. To quantify proliferation, a stacked image was taken of each annulus that contained BrdU⁺ cells, and the stack was then collapsed to produce a single two-dimensional image of each annulus. Each annulus was then scored positive for the presence of BrdU⁺ cells in either epithelial patches or ORN clusters. An epithelial patch was defined as a group of at least 10 cells in the epithelium proximal to the existing population of ORNs. To be considered a patch, these cells had to exist on the same horizontal plane, as determined by confocal microscopy. An ORN cluster was defined as any multilayered group of cells. Clusters in the mature region are composed of about 300 ORNs and associated glial cells [as described in Fig. 1(B)], while clusters in the proliferation and maturation region [Fig. 1(C)] have cells in different developmental stages. Measurements of the number of annuli that contained either patches or clusters were obtained, and mean values were calculated for antennules at each time period (1, 3, 7, 9, 12, 19, and 34 days postamputation). Statistica software (StatSoft Inc., Tulsa, OK) was used to determine statistical significance of the results.

Paraffin Sectioning

To examine the nature of cell proliferation in more detail, hemisections processed for BrdU immunocytochemistry were then embedded in paraffin and serial sectioned at 10 μ m. Tissue was dehydrated in graded series of alcohol consisting of 1-h incubations in (% ethanol) 30, 50, 70, 90, 100, 100, 100 dry ($\times 2$) (100 dry is absolute ethanol with drying beads), and cleared in 3 \times 20 min incubations in xylene. Infiltration with paraffin was done by a series of 30-min incubations in increasing concentrations of paraffin. This was achieved by continually replacing approximately half the volume of the paraffin/xylene mixture with fresh paraffin. Infiltrated tissue was then aligned in molds and allowed to set at room temperature. Blocks of tissue were then sectioned at 10 μ m using a Reichert-Jung microtome. Ribbons of tissue were floated in a water bath, collected on SuperfrostTM slides (VWR, West Chester, PA), and allowed to dry for several days at room temperature. Tissue was then dewaxed by incubating 3 \times 20 min in xylene and mounted using Cytoseal mounting medium.

Semithin (Epon) Sections

Antennules were cut into small pieces as described above, fixed overnight in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.2 M phosphate buffer (pH 7.4), rinsed in buffer, dehydrated in a graded ethanol series, transferred to acetone, and infiltrated with Eponate 12 resin (Pelco, Redding, CA). Semithin (1.0 μ m) sections were cut on an ultramicrotome using a diamond knife (Microstar, Huntsville, TX), collected on 12-well slides (Erie Scientific, Portsmouth, NH), stained with toluidine blue, and viewed and photographed using a Zeiss compound microscope.

RESULTS

Normal Growth and Development

We recently described the process of proximal addition and distal loss of olfactory sensory units in lobsters (Fig. 1) (Steullet et al., 2000a; Harrison et al., 2001a, b). ORN addition is the result of continuous cell proliferation that occurs immediately proximal to the existing ORN population [Fig. 1(E)], and the rate of this proliferation varies over the molt cycle (Harrison et al., 2001b).

In this study, we used semithin sections to examine more closely the early development of ORNs. This appears to involve a sequence of mitotic events that begins in the epithelium to produce multilayered clusters of cells, and continues within these multilayered cell clusters until the ORN clusters are fully formed. Figures 2 and 3 show examples of tissue at three different locations in the proliferation and maturation regions of the antennule, which highlight structural changes associated with this development. Proximal to the aesthetascs, the inner ventral margin of the lateral flagellum consists of a relatively flat epithelial layer composed primarily of cuboidal epithelial cells [Figs. 2(A,B) and 3(B)]. Early clusters begin to develop proximal to the existing and developing ORN population [Fig. 2(C,D)]. General characteristics that distinguish early and late stage developing clusters are position, cluster size, and organization with respect to rows. As clusters mature, their size increases and they form orderly rows [Fig. 2(E,F)]. Early stage clusters develop adjacent to the epithelium whereas later stage clusters are located clearly above the epithelium [Fig. 2(D,F)]. Furthermore, while classes of cells cannot be distinguished at early stages of development based solely on BrdU-labeling, relative positions of cells in more developed clusters enable a distinction to be made between ORNs and auxiliary (glial) cells [Fig. 2(D,F)].

Proliferation associated with this developmental sequence was examined using the cell proliferation marker BrdU. Incorporation of BrdU is most obvious within clusters [Fig. 3(A,C,D)]. However, proliferation is also seen among several other types of cells including those within the epithelium immediately proximal to the developing clusters [Fig. 3(A,B)]. Proliferation within the antennular epithelium appears to be an early step in the genesis of an ORN cluster. While confirmation of the identity of these epithelial cells will rely on the discovery of cell stage specific markers, we presume that at least some are precursors for ORNs and other cells associated with the olfactory sensory unit. Indirect

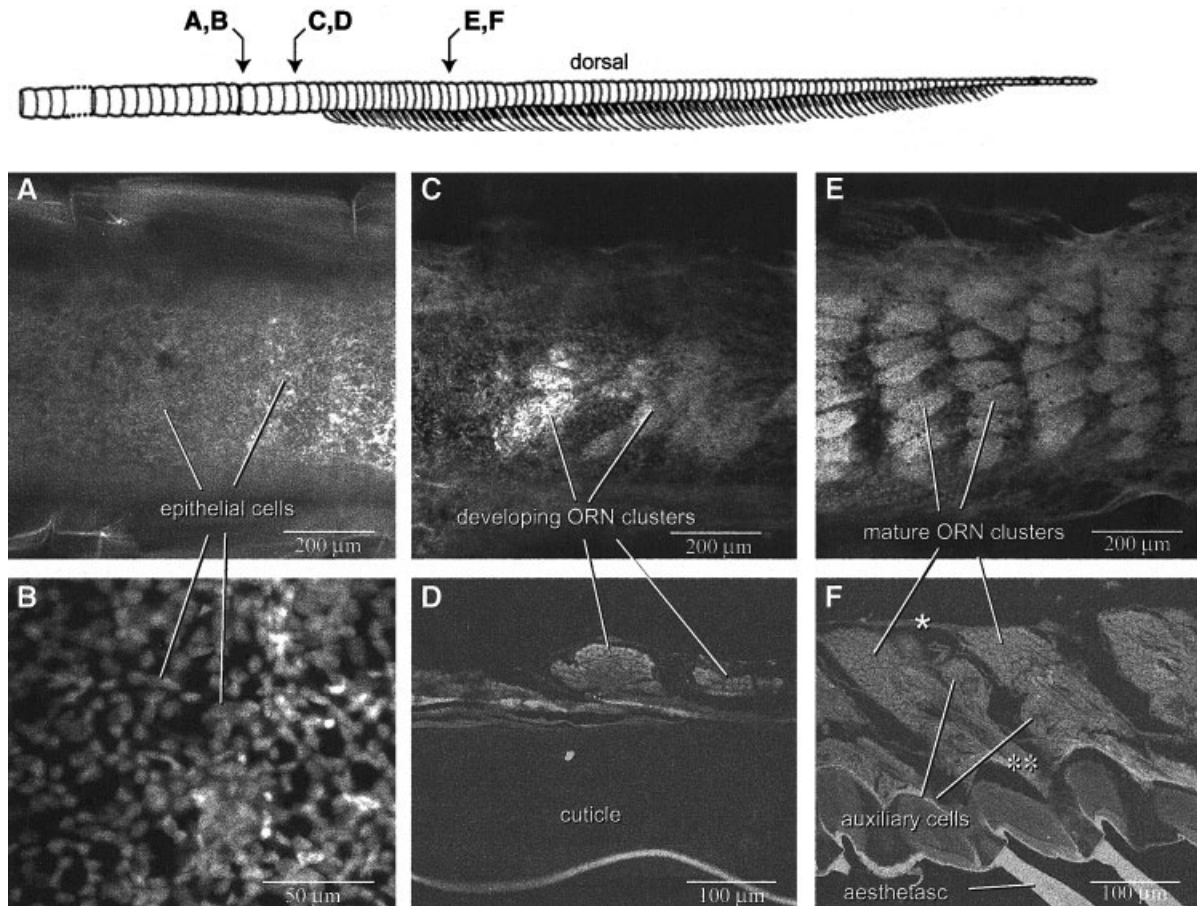


Figure 2 Morphological changes associated with postembryonic development of cell clusters. Dorsal (A–C, E) and longitudinal (D, F) views of antennular tissue. (A, B) The ventral antennular epithelium immediately proximal to the ORN population is devoid of any multicellular structures. However, proliferation that begins within this epithelium appears to be an early step in cluster development. (C, D) Clusters located proximal to the ORN population are in early stages of development. (E, F) Mature ORN clusters. Mature clusters are aligned in orderly rows that can be seen when viewed from above (E). Note this orderly arrangement compared to the scattered arrangement of developing clusters (C). (F) The organization of ORNs, auxiliary (glial) cells, axons (*), dendrites (**), and aesthetasc sensilla is apparent when viewed from the side. (A–C, E) are hemisections; (D and E) are 10 μm paraffin sections. All tissue is stained with Neurotrace (Molecular Probes, Eugene, OR), which labels all types of cells in this tissue.

support for this idea is provided by the results of experiments presented below, in which mitotic activity in the antennular epithelium is shown to be up-regulated during regeneration of olfactory sensory units. Sections taken through the proliferation and maturation regions reveal proliferation within clusters at various stages of development [Fig. 3(A,C,D)]. At early stages, bilayers of cells located immediately above the epithelium form as a result of localized cell proliferation [Fig. 3(C)]. These clusters then enlarge adjacent to the epithelium as a result of further cell division [Fig. 3(D)]. The position of the clusters shifts more vertically as de-

velopment progresses. This shift may be the result of additional cluster development that occurs more proximally [Fig. 3(D)]. Alternatively, it may occur as axons and dendrites of the developing ORNs extend to their final positions.

Development of Olfactory Sensory Units Following Antennular Amputation

Addition and loss of aesthetascs were measured in order to investigate the effect of antennular amputation on development of olfactory sensory units. Previous studies on lobsters have shown that the number

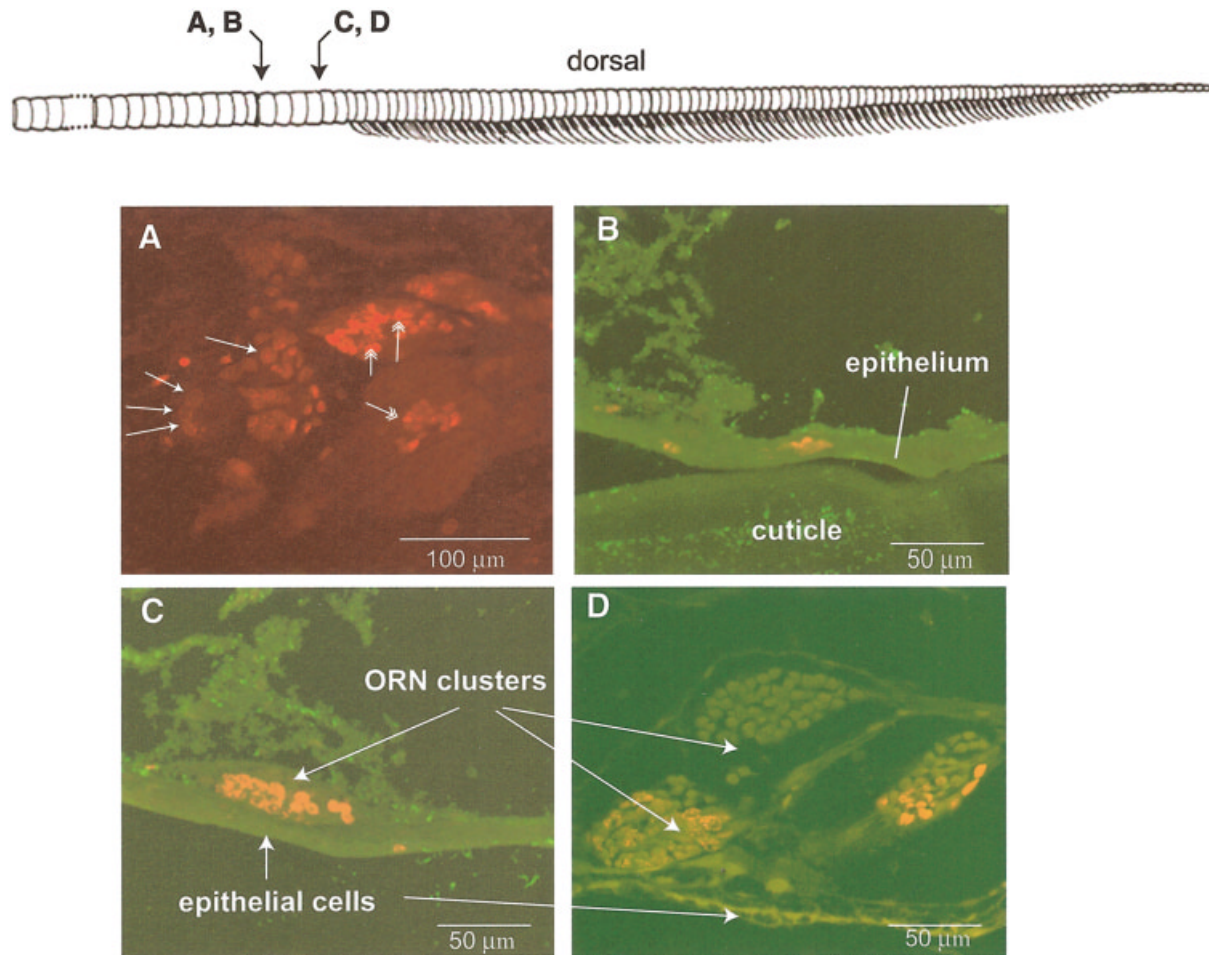


Figure 3 Proliferation associated with cluster development. BrdU⁺ cells are labeled red; green is autofluorescence. (A) Dorsal view of clusters at early stages of development and proliferating cells in the antennular epithelium from an antennular hemisection preparation. Note that nuclei of mitotically active epithelial cells (single arrows) are generally larger than those in clusters (double arrows). (B–D) 10 μm longitudinal paraffin sections. (B) Proliferation within the epithelium is apparent when viewed laterally. Note that in this area, there are no multicellular structures associated with the epithelium. (C) Clusters develop as a result of localized proliferation as opposed to recruitment of cells. (D) Clusters develop continuously proximal to existing clusters and appear to “push” more distal clusters vertically away from the epithelium.

of aesthetascs increases with each molt (Steullet et al., 2000a; Harrison et al., 2001a). This occurs in conjunction with turnover and involves proximal addition and somewhat lower levels of distal loss (Sandeman and Sandeman, 1996; Steullet et al., 2000a; Derby et al., 2001; Harrison et al., 2001b). The results of the present study indicate that addition is significantly up-regulated and loss is down-regulated following amputation of the antennule (Fig. 4). In these experiments, the left antennule remained intact and served as a control, while antennular amputation was used to remove about 80% of the olfactory sensory units from the right antennule. Left (intact) antennules on average added 14 and lost 10 aesthetasc-bearing annuli at

the molt, giving a mean net increase of four aesthetasc-bearing annuli, or approximately 60 olfactory sensory units. This approximation is based on the fact that aesthetasc-bearing annuli on animals of this size have two rows of about seven to eight aesthetascs per row. In line with previous studies, addition occurred proximally and loss was the result of shedding from the distal end. In contrast, right (amputated) antennules added an average of 38 and lost zero aesthetasc-bearing annuli. Thus, following amputation, there was a mean net gain of 38 aesthetasc-bearing annuli, or approximately 570 olfactory sensory units. Differences between amputated and intact sides were statistically significant (Fig. 4).

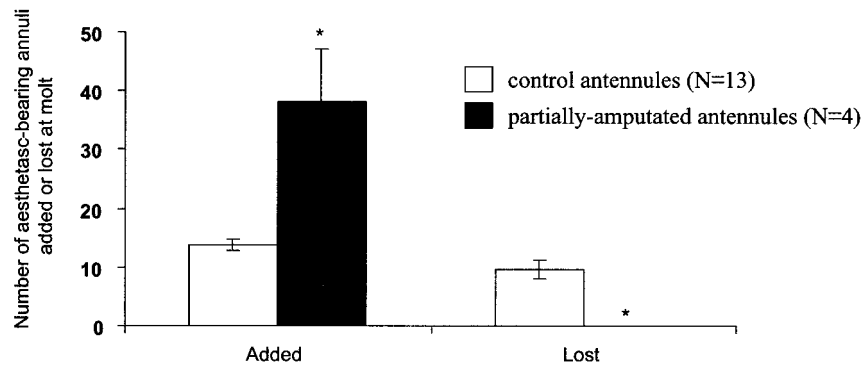


Figure 4 Changes in the number of aesthetascs added and lost at molting after partial antennular amputation. Values are mean \pm S.E.M. Asterisks represent significant differences between control and partially amputated antennules with respect to numbers of aesthetasc-bearing annuli added (Student's *t* test: $t = 4.9$, $df = 15$, $p < 0.0002$) and lost (Student's *t* test: $t = 3.3$, $df = 15$, $p < 0.005$).

Proliferation Patterns Associated with Amputation-Induced Regeneration

The experiment above highlights the significant increase in proximal addition of olfactory sensory units following amputation. Further experiments were therefore conducted to investigate cell proliferation associated with these changes. Antennules were amputated as described in the Materials and Methods section. The animals were then returned to their holding aquaria, and cell proliferation was monitored 1, 3, 7, 9, 12, 19, and 34 days later.

A significant finding of this study was that the first sign of increased proliferation was the appearance of proliferating cells in "patches" within the antennular epithelium (Fig. 5). These epithelial patches appeared between 3 and 12 days postamputation and were present predominantly on amputated sides and rarely on corresponding control sides. Most patches were easily identified; however, for the analysis presented below, it was sometimes necessary to determine whether a small number of proliferating cells located in the epithelium represented a "patch". Key features of epithelial patches used for identification using fluorescence microscopy are: patches occur proximal to developing ORN clusters [Fig. 5(A,B)]; two separate patches typically occur within the borders of each existing annulus [Fig. 5(A,B)]; the nuclei of cells in patches are typically larger in diameter than surrounding cells and particularly those in ORN clusters (see below); and proliferating cells within patches are clustered together on the same vertical plane (i.e., they are part of a uni-layered epithelium). This feature was determined using confocal microscopy and enabled patches to be distinguished from bilayers and early

stage clusters, in which dividing cells occur in two or more layers [Fig. 3(C,D)].

There is considerable variation in the size and shape of nuclei both in epithelial patches and clusters, suggesting that multiple cell types may be present in both (Figs. 3 and 5). Nevertheless, nuclei of cells in epithelial patches are typically larger than those in clusters, and there is a tendency for cell size to decrease as clusters mature (Fig. 6). Statistical analysis confirmed that cell size was significantly affected by location and developmental stage (i.e., in epithelial patch, bilayer, early cluster, and late cluster), and that all four of these groups were significantly different from each other except bilayers versus early clusters. Interestingly, cells in patches are twice as large as those in clusters. Patch-cell morphology was examined in more detail by further processing patches for semithin sectioning, but the increased resolution afforded by this method failed to reveal any further discernible features. Similar to ORNs, patch-cells have a thin strip of cytoplasm and a prominent nucleus. The cytoplasm of cells in patches does not appear to differ from that of ORNs in terms of density or its granular nature. The box plots of Figure 6 indicate that 75% of cells in late clusters have a nuclear diameter of 4.0–5.0 μm , but 25% have larger diameters of 5.0–6.0 μm . This might reflect the differentiation of cells into ORNs or different types of auxiliary (glial) cells at this later stage of development, or possibly that most, but not all, cells in these clusters have undergone their terminal division. Because of the high degree of tissue shrinkage that occurs with the use of ethanol based fixatives, cell diameters reported in the present study should be

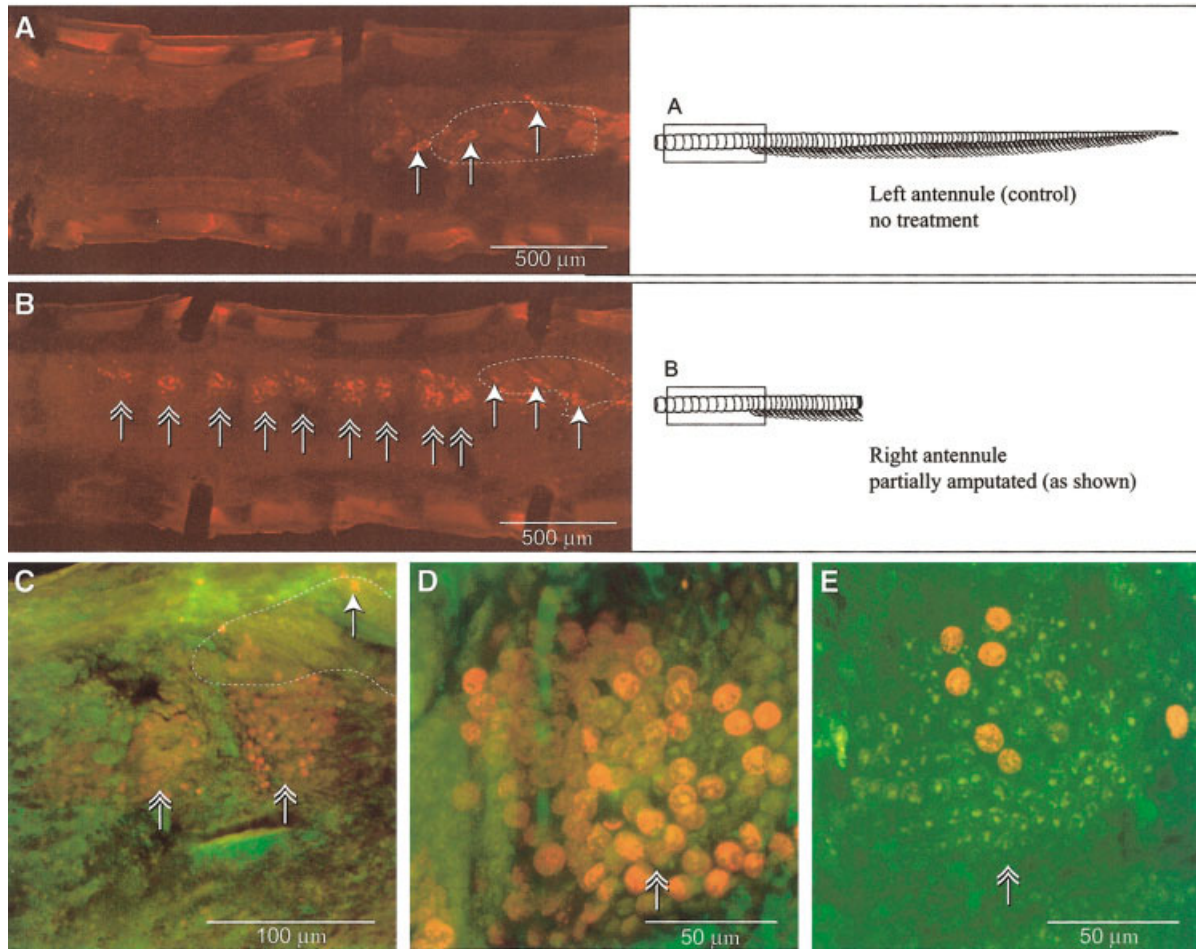


Figure 5 Examples of patches of proliferating cells appearing in the antennular epithelium shortly after antennular amputation. BrdU⁺ cells are red; tissue in (C–E) was counter-stained with Neurotrace (Molecular Probes), which highlights all cells in this tissue. (A,B) Hemisections of the left (A) and right (B) antennules from an animal processed for cell proliferation 7 days after antennular amputation, as described in the text and depicted to the right of panel (B). Dashed lines in (A–C) indicate the proximal boundary of existing (postmitotic) cell clusters. (A) Normal levels of proliferation (single arrows) occur on the proximo-lateral margin of existing cell clusters in undamaged antennules. (B) Normal levels of proliferation (single arrows) also occur on the proximo-lateral margin of existing clusters following amputation, in addition to significant levels of proliferation in the epithelium (double arrows) proximal to the existing cell clusters. (C) Higher magnification image (from a different preparation) of two epithelial patches (double arrows) adjacent to the most proximal cell cluster (single arrow). (D) Most of the cells in this patch are BrdU⁺. (E) Few cells in this fully-formed patch are BrdU⁺. Together, (D) and (E) suggest that genesis of patches can be very rapid.

interpreted in relative and not absolute terms. Cell diameters measured in mature (normal) clusters did not differ significantly from those in late clusters (i.e., approximately 5 μm, data not shown). However, in a separate study performed in our laboratory and using osmotically balanced fixative, ORN cell diameter of equivalent (mature) cells was measured at 8–10 μm (Steullet et al., 2000a).

Development of Epithelial Patches—Timing and Location following Amputation

Epithelial patches were typically observed in antennules following partial amputation. Patches were not observed in pilot studies when amputation removed all aesthetasc-bearing annuli. In general, these patches

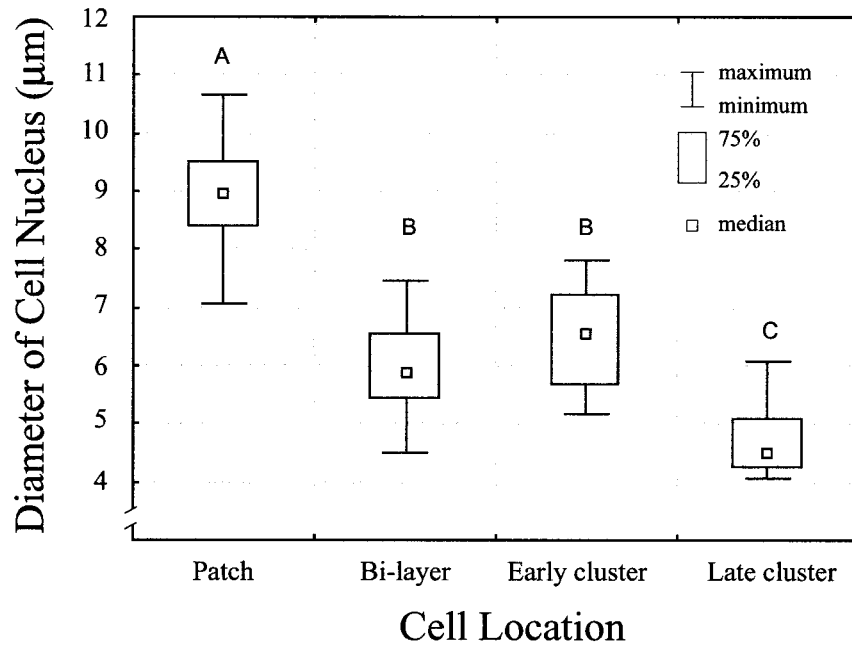


Figure 6 Quantitative changes in cell (nuclear) size during development. Values are median (small square) \pm 25% and 75% quartiles (in boxes) or range (in brackets) of nuclear diameters for 15 cells in each of four developmental stages or locations (patch, bilayer, early cluster, late cluster). Cell sizes differ significantly depending on location (Kruskal-Wallis ANOVA: $H = 44.28$, $df = 3$, $n = 60$, $p < 0.00001$). Posthoc tests reveal that only two locations—bilayers and early clusters—contain cells whose sizes do not differ significantly from each other (as indicated by letters over the bars, $\alpha = 0.05$).

were observed on amputated sides (right) and not on control sides (left) (samples taken at 1, 3, 7, 9, 12, 19, and 34 days). However, there were several exceptions, which are described in the following section. Epithelial patches were first observed at 3 days post-amputation and were most prevalent 7 and 9 days postamputation [Fig. 7(A)]. No patches were observed at 19 days after amputation, presumably because patch proliferation was complete before this time. However, after 34 days, two of the three animals tested had entered the premolt stage of their molt cycle, and significant amounts of proliferation were observed. This included proliferation of cells in both patches and clusters [Figs. 7 and 8(C)]. A two-factor ANOVA using as factors treatment [ablation (right antennule) vs. control (left antennule)] and time after amputation (weeks 1–5) showed that both treatment and time had a significant effect on patch formation ($p < 0.05$).

The effect of treatment alone on patch formation was highly significant [$F(1, 47) = 7.5$; $p = 0.009$], with patches very rarely seen on left (control) sides, except in the group tested 13 days postamputation [Fig. 7(A)]. This is consistent with observations that patches were rarely seen under normal conditions, but

it also raises the intriguing possibility that damage to one side may have had a delayed influence on patch formation on the contralateral side. It is important to note, however, that this result might also be explained if undetected damage to the left antennule occurred after the controlled amputation. These issues are discussed further in the following section.

The effect of time alone on patch formation was also significant [$F(3, 47) = 3.2$; $p = 0.033$]. From Figure 7(A), it is clear that the majority of patches developed during weeks 2 and 5, and posthoc tests confirm that the number of patches at weeks 2 and 5 is significantly greater than at week 1 (LSD, $p < 0.05$). The treatment-by-time interaction term of the ANOVA approached significance [$F(3, 47) = 2.48$; $p = 0.073$], which is consistent with the presence of large numbers of patches on the amputated side during weeks 2 and 5 but not during weeks 1 and 3.

Development of ORN Clusters—Timing and Location Following Amputation

Previous studies from our laboratory have shown that proliferation within ORN clusters occurs continuously

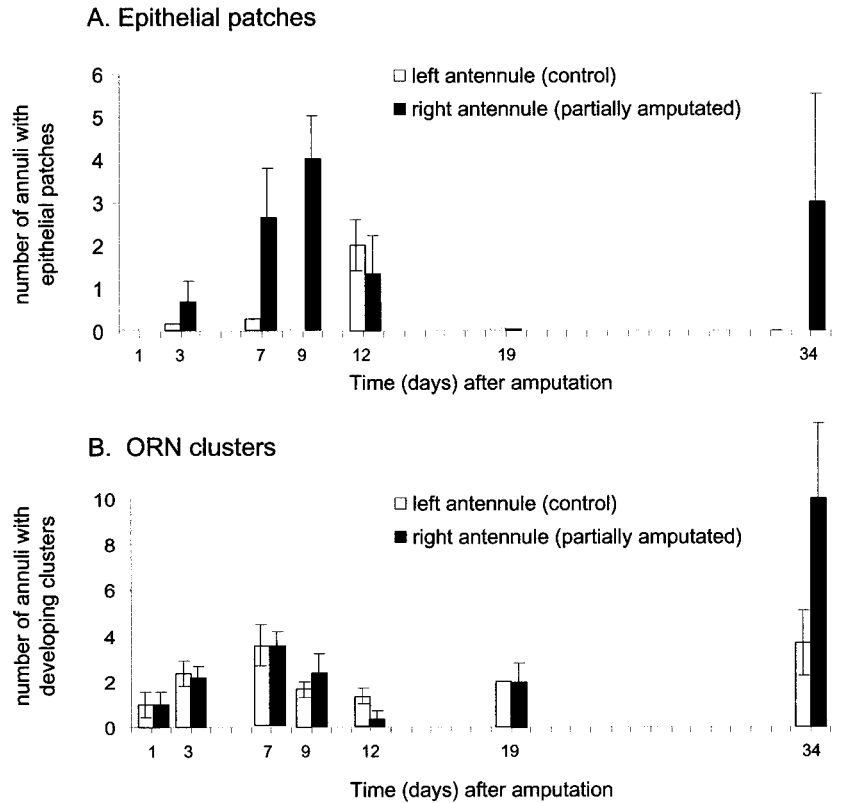


Figure 7 Quantitative changes in the appearance of proliferating epithelial patches (A) and proliferating ORN clusters (B) at different times following amputation. Values are mean \pm S.E.M. (A) There is a significant difference in the time course of appearance of patches on left versus right sides. Patches appear on right side within 3 days postablation, and the number of patches peaks after 7–9 days. A second peak in number of patches is seen after 34 days, which may be linked to molt cycle (see Discussion). (B) The number of ORN clusters does not differ between left and right sides until day 34 postamputation. This time delay probably reflects the time required for specification and activation of precursor cells in the epithelium. It is also linked to the molt cycle of these animals (see Fig. 8 and Discussion).

in adult lobsters (Harrison et al., 2001b), and this result is further confirmed here by observations of cluster proliferation at all time periods tested [Fig. 7(B)]. Cluster proliferation occurred on both amputated and control sides. A two-factor ANOVA using as factors treatment [ablation (right antennule) vs. control (left antennule)] and time after amputation (weeks 1–5) showed that both treatment and time had a significant effect on cluster formation ($p < .05$). For the treatment effect, there were significantly more ORN clusters on the amputated versus control antennules [$F(1, 47) = 4.26$; $p = 0.044$]. The effect of time was also significant [$F(3, 47) = 8.07$; $p = 0.0002$]. Posthoc tests showed that the number of ORN clusters at week 5 was significantly greater than for all other weeks (LSD, $p < 0.05$), while the other weeks were not significantly different from each other ($p > 0.05$). This is also evident from the treatment-by-time inter-

action term, which was significant [$F(3, 47) = 3.72$; $p = 0.018$]. Examination of the plot of means [Fig. 7(B)] supports the idea that this is driven by the higher proliferation of clusters during week 5 in amputated antennules. This result is consistent with the results shown in Figure 4, in which antennular amputation led to increased addition of olfactory sensory units at molt. This effect is seen clearly in Figure 8, which shows representative preparations from time points 7, 19, and 34 days postamputation. Proliferation in clusters appears normal after 7 days, and epithelial patches are seen proximal to these proliferating clusters. At 19 days postamputation, cluster development on the proximo-lateral margin of the existing population appears normal, but there is also proliferation within a single cluster in many of the annuli proximal to the population of ORNs. At 34 days postamputation, there is significant proliferation within these

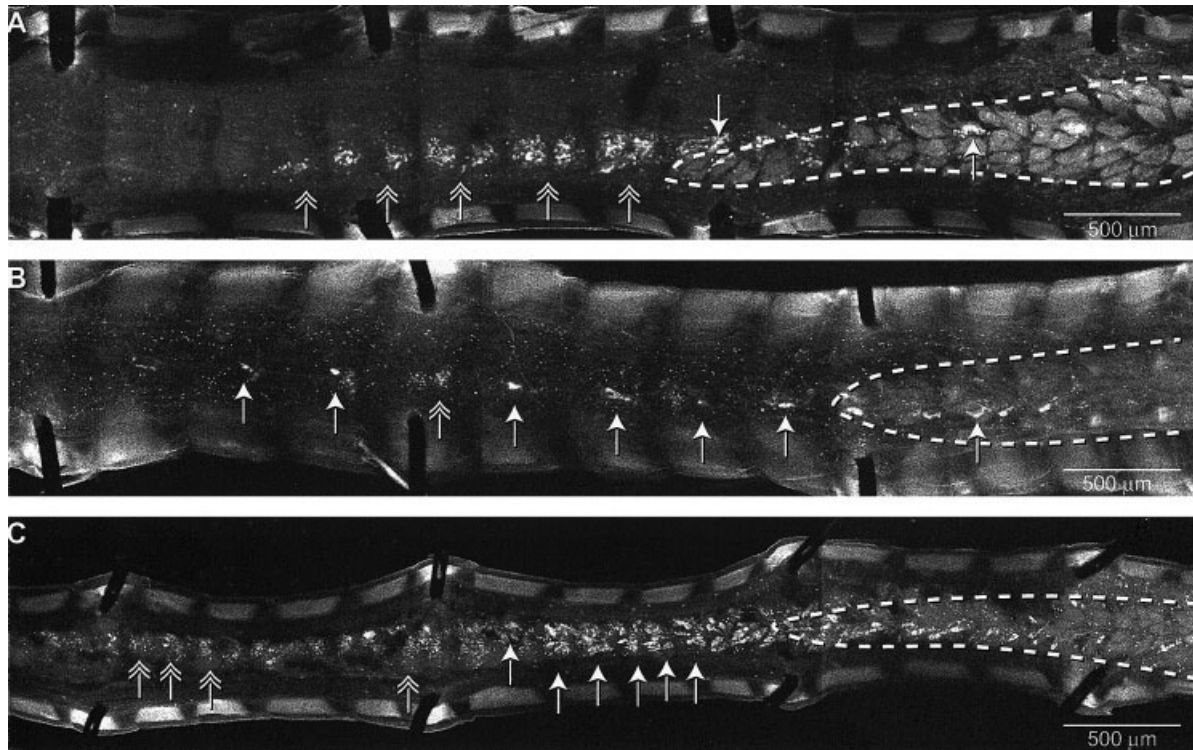


Figure 8 Pattern of proliferation at different times following antennular amputation. BrdU⁺ cells are bright. Dashed line in each figure outlines the established ORN population. (A) Cell proliferation in ORN clusters (single arrows) and epithelial patches (double arrows) at 7 days postamputation. (B) Proliferation in ORN clusters at 19 days postamputation. In this case, only two epithelial patches (double arrow) are observed, but extensive development of ORN cluster occurs in regions that would have previously contained epithelial patches. (C) There is a profound increase in proximal proliferation in ORN clusters and epithelial patches at 34 days postamputation. Panel (C) is from an animal that had entered the premolt phase of its molt cycle by the time it was injected with BrdU (see Discussion). A previous study from our laboratory (Harrison et al., 2001b) described elevated proliferation during premolt; this is particularly obvious following antennular amputation.

proximal clusters, suggesting that the majority of cell proliferation associated with regeneration of olfactory sensory units occurs during premolt.

DISCUSSION

The results show that partial damage to the peripheral olfactory system is followed by regeneration of olfactory tissue resulting from increased proximal addition of olfactory sensory units. This increased addition involves a sequence of cell proliferation that is initiated within 3 days of damage (Fig. 7). An early event in this developmental sequence is the appearance of patches of proliferating cells, located in the ventral epithelium of the antennule, immediately proximal to the existing ORN population (Figs. 3, 5, 8). These patches are induced by antennular amputation and not seen under normal conditions. Subsequent prolifera-

tion and differentiation results in each epithelial patch, yielding sets of ORN clusters and other cell types that form olfactory sensory units.

Are Epithelial Patches Composed of Multipotent Precursors?

The crustacean antennule is a continuously developing and self-renewing organ (Sandeman and Sandeman, 1996; Steullet et al., 2000a; Harrison et al., 2001a, b). It therefore requires a source of precursor cells. Similar examples of self-renewing organs include the vertebrate olfactory epithelium (Farbman, 1992; Gordon et al., 1995; Mumm et al., 1996; Huard et al., 1998; Murray and Calof, 1999; Higgs and Burd, 2001) and the ciliary marginal zone of the vertebrate retina (Sanes et al., 2000). In the vertebrate retina, the activity of multipotent precursors creates a spatial developmental gradient in which the least determined

stem cells are aligned peripherally, and fully differentiated cells that have stopped dividing are aligned centrally (Dorsky et al., 1997; Perron et al., 1998; Sanes et al., 2000). In lobsters, patch formation and subsequent histogenesis of olfactory sensory units [composed of ORNs, auxiliary (glial) cells, and aesthetasc cuticular sensilla; Fig. 1] appear similar in many ways to the developmental sequence observed during vertebrate retinal development. It is therefore likely that epithelial patches in lobsters represent a population of multipotent precursors.

These putative precursors would be expected to produce all cell types unique to aesthetasc-bearing annuli. The aesthetasc-bearing annuli differ from other antennular annuli in that they contain a complement of sensilla that is effectively superimposed on a "generalized" annulus structure (Cate and Derby, 2001). This unique complement consists of aesthetascs, guard, companion, and asymmetric sensilla (Fig. 1) (Cate and Derby, 2001). Two key observations link epithelial patches with this complement of sensilla. First, when patches occur, they typically consist of two discrete cell populations within the borders of existing annuli, reflecting the two rows of aesthetascs per annulus [Fig. 8(A)]. Second, patches have been observed only following amputation that eliminates a portion of the aesthetasc-bearing annuli but leaves the proximal proliferation zone intact. In pilot studies, patches were not observed in the medial flagellum following amputation of its outer two-thirds, or in the lateral flagellum following amputation of all of its aesthetasc-bearing annuli. Thus, patches appear to be linked specifically to restoration of aesthetasc-bearing annuli.

If the activity of precursors in epithelial patches is linked to the development of olfactory sensory units and associated tissue, then it is important to consider why patches are not seen in normal (i.e., nonamputated) antennules. In normal antennules, new tissue is continuously added as a result of the proximo-lateral progression of the proliferation zone (Harrison et al., 2001b). New clusters develop continuously on the proximo-lateral margin of the existing population of ORNs, and the rate of addition increases significantly during premolt (Harrison et al., 2001b). Thus, under these conditions, precursor activity would be expected to underlie progression of the proximal proliferation zone. While it is difficult to identify epithelial patches *per se* in undamaged antennules, it is possible to identify small numbers of proliferating cells within the antennular epithelium [Fig. 3(A,B)]. It is reasonable to assume that patches are absent under normal conditions because "normal" specification and division of precursor cells is a continuous and gradual

process that proceeds at a moderate rate. It would follow that patch formation results from up-regulated activity of these cells (Figs. 7 and 8). Current support for this idea comes from the fact that a low level of proliferation is often observed within the antennular epithelium immediately proximal to the existing ORN population [Fig. 3(A)]. Furthermore, bilayers and clusters [Fig. 3(C,D)] appear to develop as a result of proliferation that is initiated within the epithelium. The discovery of cell stage specific markers for this system will greatly help in identifying specific cell types and understanding both the various stages and temporal aspects of this developmental lineage.

Stem Cells and Restoration of Antennules

Lobsters and other crustaceans have a remarkable capacity for regeneration. Their olfactory systems undergo continuous development and have the capacity for self-renewal (Sandeman and Sandeman, 1996; Steullet et al., 2000a; Harrison et al., 2001a, b). This self-renewing capacity is in fact a hallmark of the peripheral olfactory system in many animals (Graziadei and Monti-Graziadei, 1978; Hinds et al., 1984; Chase and Rieling, 1986; Mackay-Sim and Kittel, 1990; Farbman, 1992; Murray and Calof, 1999). However, an interesting difference between lobsters and several other classes of animals is that the capacity for regeneration in lobsters is not limited by the degree of damage to peripheral stem/precursor cells of the olfactory system. This contrasts with observations from studies on mice, suggesting that damage to the neuronal colony forming cell is believed to restrict regeneration (Mumm et al., 1996; Murray and Calof, 1999).

The fact that a fully functional antennule will regenerate after complete antennular amputation indicates that stem/precursor cell populations are renewable in lobsters. Furthermore, the fact that the regenerated antennule is restored to its "correct" shape and length implicates pattern-control and quantitative-control mechanisms. This must involve regulation of a process set in motion by limb loss. Interestingly, this process can also be initiated under certain other conditions. One example is the formation of heteromorphic antennules in response to damage to the compound eye, which can be identical to the normal antennule on the same side and appear complete and functional (for lobsters and crayfish: Maynard, 1965; Maynard and Cohen, 1965; Mellon and Tewari, 2000). A second example is formation of a "third" antennule, which can occasionally develop from basal segments of the antennules following basal

segment damage that does not result in loss of the antennule (for POI stage larval crayfish: DeF. Mellon, personal communication). These results suggest that all elements necessary for formation of an antennule—neural progenitor cells and others—are present at the base of the antennule and at the base of the eye, and that these elements can be induced to proliferate under certain conditions.

This type of regulation of pattern and size of completely regenerated antennules may be explained by the polar coordinate model proposed by French (1984) for both vertebrates and invertebrates. In this model, cells have assigned positional values, and distal transformation produces cells with more distal positional values whenever cells are exposed at an amputation site. The model provides a useful explanation of limb regeneration in urodeles, in which a mesenchymal blastema controls the respecification of cell types and replacement of appropriate proximo-distal structures (Brockes, 1997). A similar process could explain both regeneration of lobster antennules and the development of heteromorphic or additional antennules as discussed above.

If this is the case, it would be useful to know the signals responsible for inducing activity in such a structure. For example, what determines the extent of patterning in the blastema? How is this patterning regulated to ensure an antennule of the appropriate size? Lobsters display highly variable growth rates, and body (and limb) size does not correlate well with age. This argues against age as a determining factor for the regulation of regenerating limb size. However, antennule size is closely correlated with body size in lobsters (Harrison et al., 2001b), implying that body size may regulate the size of regenerating antennules. How body size is translated into signals controlling pattern formation is not known, but numerous genes and transcriptional regulators are undoubtedly involved. During embryonic development in arthropods, antennules and other appendages grow from imaginal discs, which are hollow sacs of cells that make adult appendages during metamorphosis. There is evidence for the assignment of positional values in imaginal discs, and cells derived from these discs have been shown to belong to lineage compartments correlating with adult structures (Held, 2002). A genetic basis for quantitative control mechanisms underlying this process is exemplified by the activity of the *Drosophila* gene *expanded* (*ex*). This gene encodes a protein associated with apical junctions of epithelial cells and thought to regulate cell proliferation. Studies have shown that mutant flies that do not express *ex* protein have enlarged and disproportionate wings

(Boedigheimer and Laughon, 1993; Boedigheimer et al., 1997).

An anomaly such as a heteromorphic antennule presumably involves disruption to normal signaling processes, and a possible explanation for formation of a heteromorphic antennule involves transcription factors. Eye ablation may remove transcriptional regulators that would normally influence neuronal stem activity. In the absence of these regulators, other signals would bias stem cell activity to produce a heteromorphic antennule. This could be by a process similar to transdetermination, in which transcriptional regulators cause cells to switch from one determined state to another (Kalthoff, 2001). An example of this is in insects, where an eye imaginal disc, when removed from its normal developmental environment, can form a different type of appendage such as an antenna (Gehring, 1968; Kalthoff, 2001). This is consistent with the idea, derived from studies of both hematopoietic and nervous systems, that multipotential stem cells can be induced by appropriate soluble factors to yield all the adult cell lineages of a certain tissue (Anderson, 2001).

Restoration of Olfactory Sensory Units

The present study focused on restoration of olfactory sensory units, which cannot be explained easily by the polar coordinate model. In these experiments, the distal portion of the antennule was removed and a blastema formed at the amputated site. This blastema became the new tip of the antennule and distal transformations did not occur at this site. Instead, proximal transformations occurred (in the proximal proliferation zone) to compensate for the distal loss. Presumably, the extent of proximal transformation that occurs under these conditions is signaled by factors linked to the size of the animal, given that a “correct-length” antennule is always restored. In contrast to the situation for total limb regeneration, replacement of olfactory sensory units appears to rely on peripherally located control.

So where do the stem cells that give rise to peripheral olfactory tissue reside in lobsters, and how are these cells affected by damage? Both regular and damage-induced development of olfactory sensory units appear to rely on progenitor cell activity in the proximal proliferation zone (PPZ). Progression of the PPZ most likely involves either specification of new stem cells from non-neural (epithelial) tissue, or the migration of stem cells from elsewhere in the body. We favor the former idea, because olfactory development in lobsters appears to rely on proliferation that is initiated in the epithelium (Fig. 3).

An important question to address is whether latent stem cells are present in the epithelium and activated by the progressing PPZ, or whether stem cells themselves are being continuously specified from non-neural tissue. Insight into many of these issues will rely on discovery of cell stage specific markers and identification of key developmental genes. It is worth noting that progress has recently been made along these lines through the discovery of 16 mRNA transcripts with enriched expression in the PPZ (Stoss et al., 2001, 2002; Cobb et al., 2002). Further work in this area is expected to shed light on cellular and molecular processes underlying this development.

Time- and Molt-Dependent Factors

The lobster's developmental response to amputation appears to be weighted by a combination of the extent of tissue loss and the timing of loss with respect to molt stage. Several weeks are required to carry out the amputation-induced developmental sequence of epithelial patch formation followed by initial genesis of clusters and the subsequent molt-cycle dependent development of a fully differentiated olfactory sensory unit. It follows that repair within a molt cycle will be more easily achieved if damage is minimal or if it occurs early in the cycle. From the present study, patch development in the first 2 weeks after amputation most likely reflects an increase in stem cell/progenitor cell activity required for increased addition of olfactory sensory units during the molt cycle. In some cases, we observed a second phase of patch development during premolt (which in this animal was 5 weeks postamputation), which we interpret as representing a cell specification and division required for increased addition during the subsequent cycle. An intriguing implication of this is that developmental mechanisms preset the extent of recovery that will occur in each molt cycle.

Local and Centrifugal Factors

The results show that amputation-induced development is restricted largely to the ipsilateral side (Figs. 4 and 7), suggesting that local signaling mechanisms play a key role in modulating this response. Putative signaling mechanisms are outlined in Figure 9 and are described below. The fact that patches were occasionally observed on control sides leaves open the possibility that signals released as a result of damage to one antennule might induce development on the contralateral side. If this is the case, then centrifugal substances most likely play a role. However, an alternative explanation for the occasional appearance of

patches on control sides in this series of experiments is that unintended and undetected damage occurred within holding aquaria after amputations were performed.

SUMMARY AND CONCLUSION

This study confirms that an on-going sequence of cell proliferation occurs in the lobster antennule under normal conditions. It shows that the normal development of olfactory sensory units is up-regulated by antennular amputation. Complex cellular and molecular mechanisms undoubtedly govern this development. Figure 9 is a diagrammatic overview of the sequence of events reported in this study. It provides a framework upon which hypotheses relating to inductive and inhibitory signals are raised. The figure is not intended to be an exhaustive representation of possible regulatory mechanisms, but it does reflect some of our current questions on regulation of olfactory development in lobsters.

It is now clear that genesis of olfactory sensory units involves the development of several cell-types throughout the molt cycle, with the rate of proliferation being highest during premolt (Harrison et al., 2001b). Figure 9(A) depicts proliferation that occurs continuously in the antennule throughout most of the molt cycle. Low levels of proliferation are typically observed in the epithelium immediately proximal to the existing ORN clusters but not in other regions of the antennule, supporting the idea that each new sensory unit is derived from precursors in the antennular epithelium. Panels (B–D) in Figure 9 represent proliferation observed following damage to the outer portion of the antennule. This involves the sequential appearance of epithelial patches [Fig. 9(B)] and clusters [Fig. 9(C–D)], with the rate of cluster formation increasing significantly during premolt [Fig. 9(D)].

Figure 9 also shows hypothetical regulatory signals in olfactory development, excluding possible regulatory signals that act to restore full antennules following complete damage (discussed earlier). These signals include an inhibitory signal released by mature ORNs (1), an inductive signal associated with the PPZ (2), and an additional inductive signal released by the blastema that forms at the wound site following damage to the antennule (3). An inhibitory signal (1) produced by mature ORNs would be consistent with data from mammalian systems, in which the activity of underlying progenitor cells is controlled by a release of inhibition (Farbman, 1992). An inductive signal (2) is proposed in the PPZ to account for

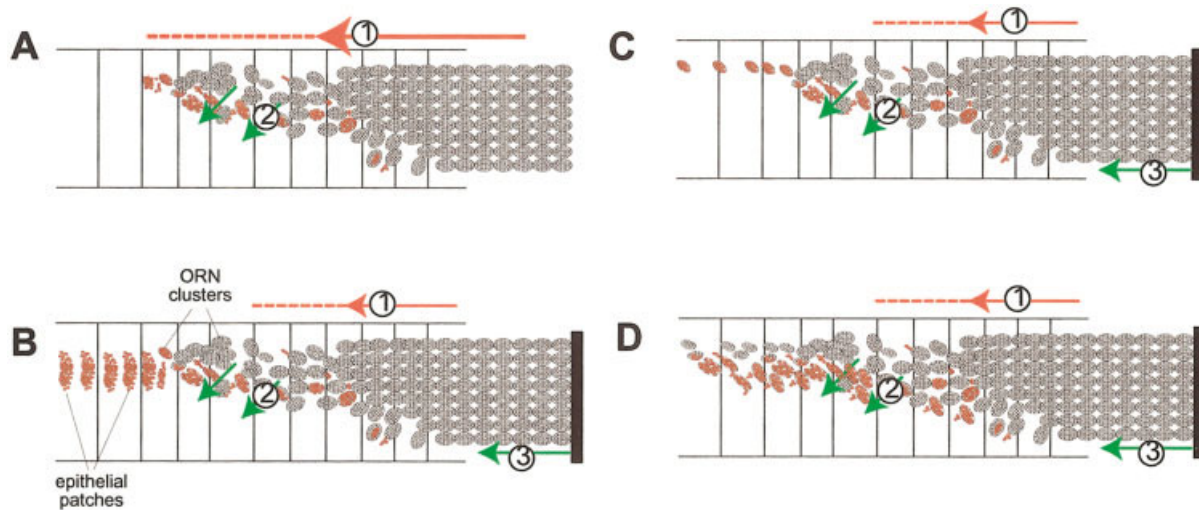


Figure 9 Diagrams to summarize ORN development under normal conditions (A) and following antennular amputation (B–D). (A) In normal (undamaged) antennules, cell clusters are continuously added to the proximal margin of the existing cell cluster population; this development appears to begin with proliferation in the epithelium [refer to single arrows in Fig. 3(A)]. (B–D) Antennular amputation (depicted by black bar) appears to initiate a sequence of cell proliferation that involves the appearance of epithelial patches (B), initial ORN cluster development (C), and rapid ORN cluster development during the premolt stage of the molt cycle (D). In these panels, 1–3 are hypothetical regulatory signals proposed to explain this sequence of events. Signal 1 is a midrange diffusible signal produced by mature ORNs, which acts on progenitor cells to inhibit or control the rate of their activity. Signal 2 is a short-range local signal produced in the proximal proliferation zone (PPZ), which opposes the activity of signal 1 and thereby promotes proliferation immediately proximal to the PPZ. The opposing action of these types of signals could be the basis for the gradual progression of the PPZ under normal conditions. (B–D) Up-regulated activity that occurs as a result of antennular amputation can also be explained by changing the ratio of these two signals (i.e., amputation lowers levels of signal 1). Signal 3 is proposed as an additional regulatory signal emerging from the damage-induced blastema. This type of signal has been reported in other systems and would be expected to induce specification and activation of precursors. These regulatory signals are presented here as hypotheses and reflect current work in our laboratory aimed at elucidating developmental regulatory mechanisms.

proximo-lateral progression of the PPZ throughout the molt cycle. If these two types of signaling mechanisms operate, then it is possible that the balance between them regulates to some degree the progression of the PPZ, and that a shift in balance toward increased inductive signal—which would occur following antennular amputation—might account for increased precursor cell activity and therefore patch formation. An additional or perhaps alternative hypothesis is that an inductive signal related to injury (3) (e.g., one produced by the blastema) is responsible for increased precursor cell activity. Either scenario is undoubtedly overly simplistic, but they outline how local signaling mechanisms might regulate this development. Either scenario provides a plausible explanation for how the level of induced activity may be linked to the degree of damage, in that greater damage would lead to a greater reduction of signal (2) and

would also increase the level of signal (3) by shifting the blastema closer to the proliferation zone.

We thank Vivian Vu-Ngo and Valentine Nduku for technical support. Spiny lobsters were provided by Lonny Anderson and the staff of the Florida Keys Marine Laboratory, Long Key, Florida.

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