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Distribution and function of *splash*, an *achaete-scute* homolog in the adult olfactory organ of the Caribbean spiny lobster *Panulirus argus*

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Abstract

achaete-scute complex (ASC) genes, which encode basic helix-loop-helix transcription factors, regulate embryonic and adult neurogenesis in many animals. In adult arthropods, including crustaceans, ASC homologs have been identified but rarely functionally characterized. We took advantage of the recently identified crustacean homolog, *splash* (*spiny lobster achaete scute* homolog), in the olfactory organ of the Caribbean spiny lobster *Panulirus argus* to examine its role in adult neurogenesis. We tested the hypothesis that *splash* is associated with but not restricted to sensory neuron formation in the olfactory organ, the antennular lateral flagellum (LF), of adult spiny lobsters. We demonstrated *splash* labeling in epithelial cells across LF developmental zones (i.e., proliferation and mature zones), in auxiliary cells surrounding dendrites of olfactory receptor neurons (ORNs), and in immature and mature ORNs, but not in granulocytes or chromatophores. Since ORN proliferation varies with molt stage, we examined *splash* expression across molt stages and found that molt stage affected *splash* expression in the ORN mature zone but not in the proliferation zone. *In vivo* incorporation of bromodeoxyuridine (BrdU) showed no correlation in the cellular pattern of *splash* expression and BrdU labeling. The intensity of *splash* labeling was dramatically enhanced in the proliferation zones following LF damage, suggesting enhanced *splash* expression during repair and/or regeneration. We conclude that *splash* is not closely associated with the formation of sensory neurons under normal physiological conditions, and we propose that *splash* is involved in repair and regeneration. We also propose that *splash* has additional roles other than neurogenesis in adult crustaceans.

Keywords

ASC homolog; sensory neuron; olfactory system; regeneration; *in situ* hybridization

INTRODUCTION

Transcription factors are essential for regulating gene expression in multicellular organisms. Spatial and temporal expression of different classes of transcription factors regulate neural development, such as cell proliferation, differentiation, and determination (Ledent and Vervoort, 2001; Bertrand et al., 2002). In recent studies, alternative functions for transcription factors classically defined in their role in development were documented. Functions include sensory maintenance (Ohkawara et al., 2004; Millimaki et al., 2010), intercellular signaling (Prochiantz and Joliot, 2003), and even interacting with AMPA/kainate receptors to regulate neuronal physiology (Chao et al., 2005).

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One of the best-studied examples of transcription factors are the *achaete-scute complex* (ASC) genes known for their role in development of cuticular sensilla of insects. ASC homologs have been identified in various animals besides insects and their roles in neurogenesis, oligodendrogenesis (Gordon et al., 1995; Cau et al., 1997, 2002; Murray et al., 2003; Manglapus et al., 2004; Kim et al., 2008), and formation of various cancers (Rapa et al., 2008; Kitamura et al., 2009) have been elucidated. ASC are basic helix-loop-helix (bHLH) genes consisting of four related types: *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*), and *asense* (*as*). In the embryonic ectoderm of *Drosophila melanogaster*, the expression of proneural ASC genes *achaete*, *scute*, and *lethal of scute* form proneural clusters that are competent to form sensillum progenitor cells (Skeath and Carroll, 1994; Bertrand et al., 2002). Up-regulation of *achaete* and *scute* in one cell of a proneural cluster induces the expression of *Notch* and *Delta*, which in turn inhibit *achaete* and *scute* expression in neighboring cells by activating *hairy enhancer of split*. This results in delamination of one sensillum progenitor cell and maintenance of ectodermal fate in the remaining cells of the proneural cluster. Sensillum progenitor cells also activate the expression of *asense*, which instructs sensillar formation (Brand et al., 1993). *asense* is expressed in all neural precursor cells (Sun et al., 1998; Wheeler et al., 2003) and thus mutations in *asense* result in broader phenotypes, suggesting that *asense* encodes a multifunctional protein (Domínguez and Campuzano, 1993).

Investigations into the identity and function of ASC homologs in crustaceans have only recently begun. Two ASC homologs have been identified in the branchiopod *Triops longicaudatus* (Wheeler and Skeath, 2005). We recently identified *splash* (*spiny lobster achaete scute* homolog) from the antennular lateral flagellum of the Caribbean spiny lobster *Panulirus argus* (Chien et al., 2009). *splash* has the conserved bHLH and C-terminal domains typical of a bHLH transcription factor, and it has higher identity with the general class of ASC homologs rather than with any one of the specific members, *achaete*, *scute*, *lethal of scute*, or *asense* (Chien et al., 2009).

The focus of our study is to elucidate the role of *splash* in the antennular lateral flagellum of adult spiny lobsters. Chemosensilla are distributed across most of the body surface of crustaceans, with the paired antennules being the most prominent chemosensory organs (Derby, 2000; Cate and Derby, 2001). Each antennule has two flagella: the lateral flagellum (LF) and a medial flagellum (MF) (Fig. 1A). The LF has two major classes of specialized sensilla: unimodal (chemoreceptive) aesthetasc sensilla, and bimodal (chemo-mechanoreceptive) non-aesthetasc sensilla of various types (Fig. 1B). Aesthetasc sensilla are limited to the ventral and distal region of the LF where they co-occur with specific types of non-aesthetasc sensilla, collectively forming a 'tuft'. The LF is comprised of pseudosegments called annuli, each containing about 20 aesthetasc sensilla. Each aesthetasc sensilla is innervated by the dendrites of approximately 300 bipolar ORNs. Associated with each aesthetasc sensilla are auxiliary cells surrounding the ORN inner dendrites (Fig. 1C) (Grünert and Ache, 1988; Steullet et al., 2000a, 2000b) and aesthetasc tegumental glands containing secretory cells arranged in a rosette (Schmidt et al., 2006). Out of the ten types of non-aesthetasc sensilla on the LF (Cate and Derby, 2001), the organization of only a few has been described (Cate and Derby, 2002; Schmidt and Derby, 2005). Axons of sensory neurons of aesthetasc and non-aesthetasc sensilla innervate the olfactory lobes and lateral antennular neuropils, respectively (Schmidt and Ache, 1992; Schmidt et al., 1992).

Adult crustaceans such as spiny lobsters which have indeterminate growth experience continuous addition and turnover of sensory systems (Schmidt, 2007). This includes continuous proliferation of aesthetasc and non-aesthetasc sensilla and turnover (Steullet et al., 2000a; Harrison et al., 2001, 2003). In addition, damage-induced loss of aesthetasc sensilla in adult spiny lobsters results in the addition of new aesthetasc sensilla, and loss of

the entire LF results in its regeneration (Harrison et al., 2003, 2004). As part of normal maintenance and growth, aesthetascs are added in the proximal proliferation zone (PZ) (Fig. 1D) of the LF (Steullet et al., 2000a). Distal to the PZ are the maturation and mature zones where ORNs mature and respond to chemical stimuli. Distal to the mature zone is the senescence zone, where aesthetascs are shed with each molt. ORN precursor cells arise as 'epithelial patches' in the ventral epithelium of the PZ, which then due to cellular proliferation form clusters of ORNs, and each cluster eventually is associated with an aesthetasc sensillum (Harrison et al., 2004). Non-aesthetasc sensilla are also added in the PZ. The rate of addition of ORN precursor cells is dependent on molt stage and degree of damage. Addition of cells is highest in premolt compared to postmolt or intermolt animals (Harrison et al., 2001). Damage to the LF increases the number of proliferating cells within the PZ, resulting in complete morphological and functional regeneration (Harrison et al., 2003, 2004; Stoss et al., 2004).

As part of our effort to understand the role of *splash* in adult spiny lobsters, we used semi-quantitative RT-PCR to examine *splash* expression in different tissues and to determine if expression levels change with molt stage (Chien et al., 2009). Our finding that the level of *splash* expression in the LF is highest in premolt animals (Chien et al., 2009), which is the molt stage when new sensilla form in the PZ (Harrison et al., 2001), is consistent with a role of *splash* in this process. However, we also found that *splash* expression levels are similar across all developmental zones of the LF, from the proliferation to senescence zones (Chien et al., 2009). Furthermore, *splash* is expressed not only in the LF but also in other neural and non-neural tissues (Chien et al., 2009). These results lead to the hypothesis that *splash* may have multiple functions in the LF of adult spiny lobster, including but not limited to participation in cellular proliferation.

Our current study aimed to test this hypothesis, using *in situ* hybridization to examine *splash* expression at the cellular level. We correlated cellular patterns of *splash* expression and adult neurogenesis in the LF, using cell type, molt stage, and LF developmental zones as variables. We found that *splash* is expressed not only in ORN precursors and immature ORNs but also in mature and non-proliferating cells of different types. Using a qualitative measure of intensity of *splash* labeling to compare expression levels in different conditions, we found that molt stage had no effect on intensity of *splash* labeling in the proliferation zone but that the mature zone of intermolt lobsters had an increase in *splash* labeling compared to premolt lobsters. We also identified an increase in *splash* labeling following damage to the LF, which suggests an association with repair and regeneration. This leads us to conclude that *splash* has a variety of functions, including novel roles, in the LF of adult spiny lobsters.

METHODS

Animals

Young adult Caribbean spiny lobsters (*Panulirus argus*) of 50–75 mm carapace length were collected near the Florida Keys Marine Laboratory and shipped to Georgia State University where they were held in 400-liter aquaria with re-circulated, filtered, and aerated artificial seawater (Instant Ocean™, Aquarium Systems Inc., Mentor, OH, USA) at 20–25°C. Animals were maintained in a 12 hr: 12 hr light: dark cycle and fed shrimp or squid three times per week. Molt stage of the animals was determined by microscopic examination of the pleopods, as described by Lyle and MacDonald (1983), and of LF after processing for *in situ* hybridization (ISH) or immunocytochemistry (ICC).

In situ Hybridization

Our protocol was a modification from those of Ishii et al. (2004), McClintock et al. (2008), and McIntyre et al. (2008).

Riboprobe preparation—We used three sets of plasmid DNA containing *splash*: a 832-nucleotide sequence for the open reading frame, a 437-nucleotide sequence for the bHLH domain of 5' *splash*, and a 387-nucleotide sequence for the C' terminal of 3' *splash* (Accession # DQ489559; Chien et al., 2009). We also cloned the binding domain of an ionotropic glutamate receptor subunit, GluR1 (Accession # GQ252689), from adult spiny lobsters and used a 433-nucleotide sequence from it (139–572 bp) for ISH. We cloned this GluR1 in *P. argus* and used it for ISH because it, like its homolog in *H. americanus* (Hollins et al., 2003; Stepanyan et al., 2004, 2006), reliably labels ORNs. Plasmid DNA sequences were digested with restriction enzymes. *GluR1* and *splash* digoxigenin (DIG) labeled antisense and sense (negative controls) RNA probes were synthesized from linearized plasmid DNA using *in vitro* transcription with DIG RNA Labeling Kit (T7/Sp6) according to the manufacturer's protocol (Roche Applied Science, Indianapolis, IN). RNA probes were precipitated by adding 4 M LiCl + 100% ethanol and placed in -80°C for 60–90 min. Probes were centrifuged at $20,800 \times g$ for 15 min at 4°C , supernatant was discarded, and the pellet was washed with ice cold 70% ethanol and centrifuged at $20,800 \times g$ for 15 min at 4°C . Supernatant was discarded and pellets were air dried and brought into solution with 25 μl diethylpyrocarbonate (DEPC)-treated distilled H_2O (dH₂O) (Invitrogen, Carlsbad, CA). Diluted RNA probes were verified using 1.5–2% agarose gels.

Tissue collection—LF were removed from animals by cutting proximal to the aesthetasc tuft region and dissected into 5–8 annuli long pieces in *P. argus* saline (460 mM NaCl, 13 mM KCl, 13.6 mM CaCl_2 , 10 mM MgCl_2 , 14 mM Na_2SO_4 , 3 mM HEPES, 1.7 mM glucose, pH 7.4, adjusted with NaOH). The annuli were numbered according to Schmidt et al. (2006), as represented in Figure 1D, with '1' representing the most proximal aesthetasc-bearing annulus, annuli proximal to '1' having progressively larger negative numbers, and annuli distal to '1' having progressively larger positive numbers. Thus, the PZ is represented by annuli -10 to 12 , with the proximal PZ being annuli -10 to -5 , the middle PZ being annuli -4 to 4 , and the distal PZ being 5 to 12 . The mature zone is represented by annuli 13 to 52 , and the senescence zone is represented by annuli 53 and greater. Dissected annuli were typically hemisectioned, thus maintaining intact the ventral aesthetasc-bearing areas. All annuli were placed in 2-ml Eppendorf tubes and fixed overnight in 4% paraformaldehyde (PFA) in 0.1 M Sörenson's buffer (SPB), pH 7.4, followed by daily incubation for 5–7 days in 0.5 M EDTA pH 8.0 for decalcification. Tissues were rinsed overnight in 0.1 M SPB and stored in fresh 0.1 M SPB at 4°C until used. Twenty-four hr before hybridization or immunodetection, annuli were embedded in 15% gelatin porcine type A 300 bloom diluted in dH₂O and fixed overnight in 4% PFA in 0.1 M phosphate buffer solution (PBS) pH 7.4 (for ISH) or 4% PFA in SPB (for ICC). Tissues were rinsed in PBS or SPB and sectioned at $50 \mu\text{m}$ using a vibratome and either placed on Superfrost Plus microscope slides (VWR, West Chester, PA) and dried on a low heat plate for ISH or placed in wells for free floating immunodetection.

Hybridization—Tissues were processed as follows: 4% PFA in PBS for 15 min, PBS (pH 7.4) for 3 min, proteinase K in Tris-EDTA (TE buffer) at 37°C for 12 min, 4% PFA for 10 min, PBS for 3 min, 0.2 M HCl for 10 min, PBS for 3 min, 0.1 M triethanol amine-HCl (pH 8.0) for 1 min with stirring, 0.25% of acetic anhydride + 0.1 M triethanol amine-HCl (pH 8.0) for 10 min with stirring, PBS at RT for 3 min, and 60%, 80%, 95%, 100%, 100% ethanol each for 90 sec. Sections were air dried, circled using a PAP pen (Electron Microscopy Sciences, Hatfield, PA), and placed horizontally in a hybridization chamber

kept moist with 50% formamide in dH₂O. Fresh hybridization solution (50% formamide in dH₂O, 10 mM Tris-Cl of pH 8.0, 200 µg/ml yeast tRNA, 10% dextran sulfate, 1x Denhardt's solution, 600 mM NaCl, 0.25% SDS, 1 mM EDTA of pH 8.0, and DEPC H₂O) was heated to 85°C for 10 min. One µl of DIG-RNA probe was added to 200 µl heated hybridization solution, mixed, and incubated at 85°C for an additional 5–10 min before being added to each slide. Parafilm was used to spread the solution and remove air bubbles before covering individual slides and incubating overnight at 55°C.

Post-hybridization—Parafilm was removed and slides were washed on a shaker as follows: 2 × sodium chloride sodium citrate (SSC), 50% formamide at 65°C for 30 min, TNE (10 mM Tris-Cl at pH 7.5, 0.5 M NaCl, 1 mM EDTA) at 37°C for 10 min, TNE + RNase A at 37°C for 30 min, TNE at 37°C for 10 min, 2 × SSC at 65°C for 20 min, 0.2 × SSC at 65°C for 20 min, and 0.1 × SSC at 65°C for 20 min.

NBT/BCIP detection—Sections were processed by treatment with DIG1 buffer (150 mM NaCl, 100 mM Tris-Cl, pH 7.5, and 1% blocking reagent from Roche Applied Sciences) at RT for 5 min, DIG1 buffer at RT for 60 min (~500 µl/slide) in a water-only moist chamber, 1:1000 anti-DIG-alkaline phosphatase (AP) (Roche Applied Sciences) in DIG1 buffer at RT for 60 min (~400 µl/slide), DIG1 buffer for 2 times in 0.01% Tween 20 at RT for 15 min each covered with foil, DIG3 buffer (100 mM NaCl, 100 mM Tris-Cl at pH 9.5, 50 mM MgCl₂) for 3 min, then in NBT/BCIP (Roche Applied Sciences) overnight in the dark at RT. The color reaction was terminated with 10 mM Tris-Cl (pH 7.5), 1 mM EDTA for 10 min and rinsed in dH₂O for 5 min. Slides were incubated in Hoechst 33258 or Sytox green (Invitrogen) for 30 min, rinsed in dH₂O, and cover slipped using Fluoromount G (Electron Microscopy Sciences). For double labeling with BrdU, tissues were transferred from slides to free-floating wells using a paint brush and continued for BrdU detection.

5-bromo-2-deoxyuridine (BrdU) Labeling

Administration—BrdU (5 mg/100 g body weight in *P. argus* saline) was injected into the hemolymph at the base of walking legs. Animals were returned to their holding tanks for 17–24-hours before tissues were collected and stored as described above.

Immunocytochemistry—BrdU detection was as follows. Sections were rinsed three times for 10 min in 0.1 M SPB and twice in 1:1 DNase I (50 mM Tris HCl pH 7.5, 10mM MgCl₂, 50 µg/ml BSA, 0.3% Triton-X-100): 0.1 M TSPB buffer (0.02% sodium azide, 0.3% Triton X-100, 0.1 M SPB) for 20 min. Sections were then incubated in 1:150 anti-BrdU antibody (mouse monoclonal, Becton Dickinson BioSciences, Franklin Lakes, NJ) diluted with antibody incubation medium (1:1 DNase I buffer and 0.1 M TSPB, 1:100 Protease Inhibitor Cocktail, 100 U/ml DNase) overnight at RT. For sections that were double labeled with BrdU, 1:200 anti-phosphorylated histone 3 (pH3) (Santa Cruz Biotechnology, Santa Cruz CA) was added. In other ICC procedures, we used 1:500 anti-GluR1 rabbit polyclonal antiserum raised against *Homarus americanus* Accession # GQ252689 (Hollins et al., 2003; Stepanyan et al., 2004, 2006) to label mature ORNs and 1:10,000 anti-acetylated tubulin mouse monoclonal antibody to label non-aesthetasc sensory neurons (Schmidt and Derby, 2005). Primary antibodies were diluted in 0.1 M SPB and left overnight at RT. All sections were rinsed in 0.1 M TSPB four times for 30 min each then incubated for 4–12 h in a secondary antibody goat anti-rabbit (1:200 CY3 goat anti-rabbit or Alexa 488 goat anti-mouse: Jackson ImmunoResearch, West Grove, PA) and on some sections the F-actin marker phalloidin Alexa 488 1:50 (Molecular Probes, Eugene OR) (data not shown) was added at 1:100 dilution. Sections were rinsed three times for 30 min each in 0.1 M SPB followed by either 0.007mg/ml Hoechst 33258 or 10⁻⁴ mM Sytox green (Invitrogen) for 30 min, then rinsed in 0.1 M SPB. Sections were mounted on slides and

coverslipped using Fluoromount G (for double labeling with *splash* RNA probe) or 1:1 glycerol/0.1 M SPB containing 5% diazabicyclol [2.2.2]octane (DABCO) and 0.01% sodium azide.

Image Analysis

NBT/BCIP-labeled tissues were analyzed using a light microscope (Axioplan2: Zeiss, Jena, Germany) and images captured using a digital camera Leica DC 500 and images were processed in Paint Shop Pro 5 (Jasc Software, Eden Prairie, MN). For fluorescently labeled tissues, a multi-photon microscope was used (LSM 510: Zeiss, Jena, Germany). An argon laser was used to visualize AlexaFluor-488 labeled secondary antibodies, a helium-neon laser to visualize CY3-labeled secondary antibodies, and a titanium-sapphire 2-photon laser was used to visualize Hoechst 33258. All digital images were arranged into figures using Adobe Illustrator CS 11.0 .0 (San Jose, CA).

Ablation Experiments

Ablations were performed as described by Harrison et al. (2003). Intermolt animals of 56–73 mm carapace length with no visible damage were taken from their aquarium and the right LF was ablated with scissors, removing ca. 80% of the aesthetasc-bearing annuli. Animals were observed for 10–15 min and returned to their holding aquarium. The remainder of the right LF containing the proliferation zone and some transition zone, and the left LF (unablated side), were collected 5, 7, or 9 days after ablation. Tissues were fixed and processed for ISH in blocks, where a block consisting of 5, 7, and 9 days post-ablation tissues was processed on the same day, and three different blocks were processed on three different days. This allowed a degree of normalization in our quantification. Images were captured as described above.

Data Analysis

The effect of molt stage on *splash* expression in the LF was evaluated by examining tissue sections from three blocks of premolt animals and intermolt animals. We rated the intensity of *splash* labeling in the proliferation zone and mature zone. ORN precursor cells in the epithelium and ORN clusters were identified as previously described (Harrison et al., 2001, 2003). Intensity of labeling was rated by an observer who was blind to the condition of the antennule. These data are presented in two ways. One presentation is based on counts of *splash*⁺ vs. *splash*[−] labeled cells in premolt vs. intermolt animals, yielding a molt-stage comparison of the percentage of labeled ORN precursor cells and labeled ORN clusters in the proliferation zone, and the percentage of labeled ORNs in the mature zone. The mean values ± SEM are presented in stacked bar graphs in Figure 4. The second method was a comparison of the intensity of *splash* labeling in ventral epithelium and ORNs in the proliferation zone and mature zone of premolt vs. intermolt animals. Intensity of *splash* labeling in this case was not based on cell counts, but on intensity of label across the tissue. Counting of cells in the ventral epithelium of pre-molt animals can be difficult since cells in this tissue are often very close together and difficult to distinguish. In this second method, intensity of labeling was quantified using the following scale: no labeling (−), faint labeling (+), intense labeling (++), and very intense labeling (+++). Median values from the three animals in each molt condition were determined and shown for the three regions of the proliferation zone (pPZ, mPZ, dPZ) and mature zone (MZ) in Table 1.

The effect of antennular ablation on *splash* expression was examined for three groups of animals (three animals per group): five-, seven-, or nine-days after ablation (P5, P7, P9, respectively). Intensity of *splash* labeling was quantified as described in the second method of the preceding paragraph, in part because the regenerating tissue makes cellular resolution within the PZ difficult, and the data are presented as the median intensity for two regions of

the proliferation zone (pPZ, dPZ) and at the site of injury of the ablation (results shown in Table 2).

The degree to which *splash* expression is coincident with proliferating cells was examined by comparing the percentage of BrdU+ cells that are also *splash*+ in the proliferation zone of three intermolt animals. The numbers of BrdU+/*splash*+ and BrdU+/*splash*- cells were counted for each animal and mean percentage values were calculated and shown in Figure 5.

Chemicals

All chemicals were obtained from Sigma Aldrich (St. Louis, MO) unless otherwise noted.

RESULTS

Cellular Localization of *splash*, an ASC Homolog, in the Antennular Lateral Flagellum

Towards understanding the role of ASC homologs in adult crustaceans, we examined the cellular expression pattern of *splash*, a spiny lobster *achaete-scute complex* (ASC) homolog. We tested the hypothesis that *splash* is associated with but not restricted to the formation of ORNs and other sensory neurons in the antennular lateral flagellum (LF) of adult animals. We localized *splash* labeling using digoxigenin RNA probes for *in situ* hybridization in adult lobsters. We first established the pattern of labeling in adult intermolt animals to compare it with premolt animals and for regenerating LF (where proliferation is highest), and also examined the correlation between *splash* expression and cellular proliferation in the LF. Adult neurogenesis associated with sensilla in the tuft region of the LF originates from proliferation in the ventral epithelium in the proximal part of the proliferation zone (= proximal PZ) (Harrison et al., 2003, 2004). If *splash* functions in sensilla formation as do other proneural ASC homologs, then we expected to detect patches of *splash* labeling in the ventral epithelium of the proximal proliferation zone, or one very intense *splash*+ cell within each epithelial patch.

We began by examining *splash* labeling in intermolt LF. The cellular morphology of the tuft region of unlabeled intermolt LF is shown in sagittal section in Figure 2A (also see Fig. 1C). The aesthetasc sensilla are on the ventral surface of the LF where ventral epithelial cells and clusters of ORNs, with their dendrites extending into the aesthetascs, are located. Dorsal to the aesthetasc-bearing cuticle is a layer of dorsal epithelial cells. We tested three digoxigenin labeled *splash* RNA probes that targeted the open reading frame, the bHLH domain, and the C' terminal (data not shown). In intermolt PZ, we found intense *splash* open reading frame RNA labeling in the cytoplasm of epithelial cells in the ventral epithelium of the proximal PZ where new ORN clusters had not yet formed (Fig. 2B). However, *splash* labeling in the epithelium was uniform and not organized as patches. In the middle PZ, most newly formed ORN clusters showed very intense *splash* labeling (Fig. 2C), but these were interspersed with ORN clusters devoid of *splash* labeling. In most of the labeled clusters, a few ORNs and some neighboring epithelial cells were unlabeled (Fig. 2D). Using *splash* 5' bHLH probe (Fig. 2E) and C' terminal probe (data not shown), labeling had a similar pattern but of faint intensity compared to the ORF probe (Fig. 2C and D). We did not detect *splash* labeling in the numerous chromatophores (Fig. 2F), which are multipolar pigmented cells that provide coloration (Noël and Chassard-Bouchaud, 2004). We also did not detect *splash* labeling in granulocytes that are located throughout the LF (Battison et al., 2003; Schmidt and Derby, 2006) (Fig. 2G).

As a positive control for our DIG labeled RNA *in situ* hybridization, we used RNA probes based on clone of a partial sequence of a *P. argus* ionotropic glutamate receptor, GluR1 subunit. Our *P. argus* GluR1 clone (Accession # GQ252689) was of the binding domain subunit, and this domain has 97% sequence identity to OET07, the GluR1 in *H. americanus*

(Hollins et al., 2003). Our *GluR1* probe labeled ORNs in *P. argus* (Fig. 2H), as expected since the *H. americanus* *GluR1* homolog also labeled ORNs (Hollins et al., 2003; Stepanyan et al., 2004, 2006). The *GluR1* probe labeled all ORNs in the PZ, including immature cells, and all ORNs in mature zone of LF but did not label any other types of cells in the LF. Immunocytochemical double labeling using anti-GluR1 antiserum and the nuclear marker Hoechst 33258 confirmed and extended results of RNA labeling, by showing labeling in the somata and dendrites of ORNs in the PZ and mature zone (Fig. 2J).

For negative controls of *in situ* hybridization, we used a *GluR1* sense probe (Fig. 2I), hybridization buffer omitting *splash* RNA probe (Fig. 2K), an RNase control in which tissue was exposed to RNase A prior to adding *splash* RNA probes (Fig. 2L), and a control lacking primary antibody anti-DIG (Fig. 2M). In these negative controls, we saw no labeling of cellular structures other than aesthetasc tegumental glands (Schmidt and Derby, 2005). Most controls showed non-specific binding in the acellular cuticle. Our prior success using a similar *in situ* hybridization procedure to localize a CUB-serine protease in the olfactory organ (Schmidt et al., 2006) also served as a positive control for our current study of *splash* labeling. One of our negative controls, *splash* sense probes, occasionally labeled epithelial cells and ORNs. We speculate that labeling with the *splash* sense probes may occur due to a *splash* natural antisense transcript, as identified for many genes (Alfano et al., 2005; Katayama et al., 2005; Mercer et al., 2008, 2010; Smalheiser et al., 2008) including one in prawn (Yin et al., 2007).

Effect of Molt Stage on *splash* Labeling

Molt stage affects the number of ORN precursor cells in that premolt animals have more BrdU+ cells compared to postmolt or intermolt animals (Harrison et al., 2001). If *splash* expression is correlated with neural proliferation, then we expect to observe lower levels of *splash* labeling in the epithelium of the PZ of intermolt animals, where the lowest numbers of BrdU+ cells are observed. To begin to test our hypothesis, we performed *in vivo* BrdU incorporation followed by ICC. We found many isolated BrdU+ cells in the proximal ventral epithelium. In the middle and distal PZ, we found BrdU+ cells in clusters (Fig. 3B). Triple-labeling of tissue with anti-BrdU, anti-phosphorylated histone 3, and Hoechst 33258 showed several M-phase (i.e. phosphorylated histone 3 +) cells within ORN clusters containing S-phase (i.e. BrdU+) cells (Fig. 3C). This suggests that neural proliferation occurs in intermolt LF, as previously described (Harrison et al., 2001).

We examined *splash* labeling pattern throughout the LF of intermolt lobsters and compared it to the LF of premolt lobsters. In proximal PZ of intermolt animals, which lack immature ORN clusters, we found intense *splash* labeling in the cytoplasm of ventral epithelial cells (Fig. 3D, Table 1). In middle PZ, we also observed *splash* labeling in epithelial cells as well as in many cells of newly formed ORN clusters, most likely representing immature neurons (Fig. 3E) (N= 4). Interestingly, some immature ORN clusters adjacent to intensely labeled clusters were not labeled (Fig. 4). We observed labeled clusters that contained some unlabeled cells (Fig. 4). Our observation of numerous *splash*+ cells in the PZ and our semi-quantitative PCR data showing that non-proliferating tissues have *splash*+ cells (Chien et al., 2009) led us to examine the distal PZ where ORNs differentiate and the mature zone where chemical-responsive ORNs are located. We found abundant and heterogeneous *splash* labeling in somata of cells in the ventral epithelium and ORNs in the distal PZ (Fig. 3F) and mature zone (Fig. 3G, Table 1). Each ORN cluster also contained unlabeled cells (Fig. 4). Aesthetasc tegumental glands, which are abundant in the aesthetasc-bearing zone, were non-specifically labeled. The mature zone of intermolt lobsters had *splash* labeling of similar intensity to that in the distal PZ (Table 1), including very intense *splash*+ cells in the ORN clusters (Fig. 4, Table 1). Our method of determining molt stage verified that our intermolt animals, as expected, did not have new cuticle and setae formed beneath the old cuticle (Fig.

3H). To determine the effect of molt stage on *splash* labeling, we examined premolt LF. Unlike intermolt LF, premolt proximal PZ contained many newly formed ORN clusters and auxiliary cells (Fig. 3I) that will become associated with aesthetasc sensilla that emerge after the animal molts. Not as many epithelial cells in the ventral epithelium of premolt animals were intensely labeled for *splash* compared with intermolt animals (Table 1). Similar to intermolt, we found some unlabeled ORN clusters interspersed with many clusters that had *splash* labeling (Fig. 4). In distal PZ (Fig. 3J) and mature zone (Fig. 3K) of premolt animals, we observed patches with intense *splash* labeling (Table 1) in the ventral epithelium proximal to each sensillum. In addition, most ORN clusters contained both *splash*⁺ and *splash*[−] cells (Fig. 4). Contrary to our prediction, we found no molt stage related differences in the degree of *splash* labeling in the ventral epithelium. In premolt animals, new cuticle and setae formed beneath the old cuticle and setae (Fig. 3L). Thus, our lack of correlation between molt stage and intensity of *splash* expression was not due to the technique of molt staging. Interestingly, we observed that *splash* labeling in immature and mature ORN clusters was more intense in intermolt animals compared to premolt animals (Table 1). The majority of ventral epithelial cells in the distal PZ and mature zones of intermolt animals also showed more intense labeling compared to premolt animals.

Because *splash* labeling was not specific to proliferating areas of the LF, we wanted to determine if it is a general feature of all cell types in the LF. We observed *splash* specific labeling in auxiliary cells surrounding the dendrites of ORNs (Grünert and Ache, 1988) (Fig. 3F, I, J) and non-specific labeling in aesthetasc tegumental glands, which are abundant in the aesthetasc region of the LF. Furthermore, we found *splash* labeling in dorsal epithelium (Fig. 3M) and in mesial and lateral regions of the LF (data not shown) where proliferation is not known to occur. Unlike in the ventral epithelium, most if not all dorsal epithelial cells contained *splash* labeling. In distal regions of the LF in regions proximal to the mature zone, there was a decrease in the intensity of *splash* labeling in dorsal epithelial cells (Fig. 3N). Granulocytes and chromatophores in LFs of intermolt (see Fig 2) and premolt (data not shown) animals did not have *splash* labeling. These data suggest that *splash* is not tightly correlated with molt stage dependent neural proliferation but is clearly involved in other functions, as indicated by labeling in mature ORNs, auxiliary cells, and dorsal epithelial cells, all of which do not normally show cell proliferation in adult animals.

***splash* is Not Exclusively Expressed in BrdU⁺ Cells**

To determine whether there is a relationship between *splash* expression and adult cell proliferation, we harvested LF 24-h after BrdU injection and examined this tissue for BrdU and *splash* labeling in the ventral epithelium of the PZ. If *splash* is involved with the generation of ORNs, then we expect that proliferating (i.e. BrdU⁺) cells will be *splash*⁺. As previously described (Harrison et al., 2001), we observed BrdU⁺ cells in several regions of the PZ, including the ventral epithelium, newly formed ORN clusters, and other areas. We also observed BrdU⁺ cells with different nuclear shapes within the PZ. The majority of BrdU⁺ cells in clusters were located in the lateral LF. We found numerous BrdU⁺ cells (Fig. 5A) and numerous *splash*⁺ cells (Fig. 5B), but very few double-labeled cells (i.e. *splash*⁺ and BrdU⁺) (Fig. 5D) in the ventral epithelium and ORN clusters of the PZ (Fig. 5B, D). The few double-labeled cells were clearly distinct from other epithelial cells based on the shape of the nucleus, which appeared more circular compared to surrounding epithelial cells that were either *splash*⁺ or BrdU⁺ (Fig. 5C). There were very few cells that had the same morphology but were *splash*⁺ and BrdU[−] (Fig. 5C). These data do not provide direct evidence for *splash* expression with cell proliferation.

***splash* Labels Non-Aesthetasc Sensory Neurons in the Lateral Flagellum**

The LF has unimodal (chemo-mechano) non-aesthetasc sensilla (Fig. 1B). We already showed *splash* labeling in aesthetasc ORNs (see Fig. 3). To determine whether *splash* is associated with non-aesthetasc sensilla, we examined the regions of the LF containing non-aesthetasc but not aesthetasc sensilla – the lateral and mesial regions of LF – for *splash*⁺ cells. We found large *splash*⁺ cells (approximately 30 μm × 20 μm L × W) of various morphologies that were not aesthetasc ORNs (5 μm) (Fig. 6A–H). These cells were found as singlets (Fig. 6C, D), pairs (Fig. 6A, B), or groups of four to six (Fig. 6E–H). The somata of these cells had bipolar extensions and a spherical nucleus containing a large central nucleolus typical of sensory neurons. Their nucleus lacked *splash* labeling and was larger than the nucleus of ORNs. The nucleolus was often *splash*⁺ (Fig. 6A, D, F). All identified large bipolar cells, unlike ORNs, were *splash*⁺ (> 200 cells, N = 6 LF). A subset of the bipolar *splash*⁺ cells appeared to send processes ventrally towards the cuticle (Fig. 6E–H). We also observed small *splash*[–] cells among the large bipolar *splash*⁺ cells (Fig. 6H). Some of the large bipolar cells were located more closely to either the lateral, mesial, or dorsal region of PZ of the LF but hardly ever in medial region where ORNs were located. These cells were found in all areas of LF, from the PZ to the mature zone and in animals of any molt stage.

Based on their bipolar morphology and position on the LF, we hypothesized that some of these cells innervate non-aesthetasc sensilla. To test this, we examined the mature zone labeled with three markers: anti-acetylated tubulin and the F-actin marker phalloidin (data not shown) to visualize non-aesthetasc sensory neurons and their processes (Schmidt and Derby, 2005); and Hoechst 33258 to visualize nuclei (N=6). We found that numerous large bipolar cells (nucleus diameter, 15 μm) were located in approximately the same anatomical position on LF as observed with *splash* labeling. Many of the bipolar cells innervated non-aesthetasc sensilla (Fig. 6I), though some in this plane of section were not clear (Fig. 6J). Unlike ORNs (nucleus diameter, 8 μm), these cells did not express GluR1 (ISH, N=10; ICC, N=2) (Fig. 6K, L). These data demonstrate that *splash* is associated with unimodal (chemo) and bimodal (chemo-mechano) sensilla in the adult olfactory organ.

Damage to the Olfactory Organ Increases *splash* Labeling

The LF exhibits remarkable plasticity. Partial or complete ablation of the LF in an intermolt animal causes a large increase in addition of aesthetascs and related sensory tissue in the proliferation zone, resulting in regeneration of the LF to the length and shape of its pre-amputated size (Harrison et al., 2003, 2004) over the course of a few months. Regeneration is fueled by a gradual increase in the number of BrdU⁺ cells in epithelial cells in the PZ that begins as early as 3 days after ablation, peaks at 9 days after ablation, and is over by 19 days post-ablation. Ablation also causes an increase in formation of BrdU⁺ ORN clusters, with one peak occurring at 7 days after ablation and with another peak just before the next molt (Harrison et al., 2003). We hypothesized that if *splash* is involved in damage-induced proliferation, then we would expect an increase in *splash* labeling on the same time course as BrdU labeling following damage. We unilaterally damaged the LF by partially ablating its distal end and examined its PZ at 5-, 7-, and 9-days post-ablation. In the proximal and middle PZ 5 days after ablation, *splash* labeling was present in some epithelial cells but not in ORN clusters, and the intensity of labeling was low (Fig. 7A, C, respectively, Table 2). At 7 (data not shown) and 9 days (Fig. 7B, D) after ablation, *splash* labeling in epithelial cells and ORN precursor cells was intense. In the distal PZ at 5 days post ablation, *splash* labeling was more intense compared to the proximal PZ (Fig. 7A) but not as intense as at 7 or 9 days post-ablation (Fig. 7 B–F, Table 2). Thus, following LF damage, *splash* expression increased in areas of the LF where cellular proliferation also occurred, and had a similar time course as the increase in cell proliferation.

To determine the role of *splash* in damage-induced repair that leads to regeneration, we examined the areas next to the site of damage (i.e., immediately proximal to the blastema), which includes the mature zone. *splash* labeling was present in the ventral epithelium at post-ablation days 5 (Fig. 7G), 7, and 9 (Fig. 7H), but the labeling was more intense and widespread at later times, including in lateral and mesial epithelial cells (Fig. 7I, Table 2). The increase in *splash* labeling compared to intermolt mature zone (see Fig. 3) suggests that *splash* is involved in damage-induced proliferation and in damage-induced repair.

DISCUSSION

This is the first description of the cellular distribution of an *ASC* homolog in an adult arthropod. *splash*, an *ASC* homolog in the Caribbean spiny lobster *Panulirus argus*, is expressed in the adult antennular lateral flagellum (LF), a major chemosensory organ in decapod crustaceans (Chien et al., 2009). Our in situ hybridization results show that *splash* is expressed in both immature and mature ORNs and also in other cell types. Molt stage, which affects the extent of ORN proliferation (Harrison et al., 2001) and the extent of *splash* expression in the LF (Chien et al., 2009), did not affect *splash* expression in proliferating ORNs but it did affect expression in mature ORNs. Damage to the LF, which induces regeneration, also increased the intensity of *splash* labeling, suggesting an association between *splash* with regeneration and repair. We conclude that *splash* plays multiple roles in the adult spiny lobster olfactory organ.

The Role of *splash* in Neurogenesis in the Olfactory Organ of Adult Spiny Lobsters

The function of *ASC* homologs in neurogenesis is best known from studies of the formation of sensilla in embryos of insects, especially *Drosophila*. In this model, *ASC* homologs are expressed in unspecified ectodermal cells that form a proneural cluster, from which one cell takes on a neural fate (Skeath and Carroll, 1994; Skeath and Doe, 1996) ultimately forming a sensory organ. According to this model, if *splash* is a proneural gene and functions in ORN neurogenesis of adult spiny lobsters as do *ASC* homologs of insects, then we would expect to observe one or a cluster of *splash*⁺ cells in the ventral epithelium of the proliferation zone where new ORNs originate. However, we found that *splash* is uniformly expressed in the ventral epithelium of the LF of intermolt animals (Fig. 2). *ASCI* homolog expression in crustacean larvae initially has a uniform expression pattern, but as development proceeds, selected cells within a cluster increase expression (Wheeler and Skeath, 2005) suggesting that insect and crustacean development is similar. Our results do not support the idea that *splash* functions as a proneural gene in the LF of adult spiny lobsters. Relevant to this idea are our data on the effect of molt stage on *splash* labeling. Because molt stage affects proliferation, with the number of BrdU⁺ cells in PZ being higher in premolt compared to intermolt animals, we examined the effect of molt stage on *splash* labeling. If *splash* were a proneural gene, then conditions in which cell proliferation in the PZ is enhanced, such as in the premolt stage, should also increase *splash* labeling. Counter to this expectation, we found no major difference in the intensity of *splash* labeling in the PZ of premolt and intermolt animals (Fig. 3, Table 1). Our third evidence that *splash* does not function as a proneural gene in the PZ is the labeling of epithelial cells across all LF developmental zones including a higher expression in the dorsal epithelium (where aesthetascs and their ORNs do not reside) versus ventral epithelium of the PZ.

We determined whether there is a direct relationship between *splash* expression and cell proliferation using BrdU labeling. We previously showed that after a one-time injection of BrdU and a 24-h survival time, many epithelial cells formed patches and many cells within developing ORN clusters in the proliferation zone were BrdU⁺ (Harrison et al., 2001; 2003). Using the same labeling strategy, we also found many BrdU⁺ cells in both locations; however, only a small percentage (6% for epithelial cells, 18% for cells in ORN clusters) of

these BrdU+ cells were also *splash*+ (Fig. 5). To interpret this result, two aspects of BrdU labeling have to be considered. Firstly, BrdU is incorporated into DNA during the S-phase of the cell cycle and is then distributed into the two daughter cells generated by the cell division. Thus a BrdU+ cell can be either a proliferating cell (in S-phase) or a post-mitotic cell. Secondly, BrdU can be incorporated into the DNA of proliferating cells as long as it is available in the hemolymph, which is 12–24-h post injection in another crustacean, the crayfish *Pacifastacus leniusculus* (Söderhäll et al., 2003). Given the fact that there is no evidence for a synchronization of cell divisions in the proliferation zone (Harrison et al., 2001; 2003), the population of BrdU+ cells that we observed 24-h after BrdU injection should include a sizeable proportion of cells that were still in S-phase (proliferating) at the time of fixation. Thus, the finding that only a minor percentage of BrdU+ cells in both locations was also *splash*+ suggests that expression of *splash* is not tightly correlated with cell proliferation. In conjunction with our other results, this leads us to conclude that *splash* does not play a major role in proliferation that gives rise to sensory neurons in adult spiny lobsters.

***splash* May Play a Role in Repair and Regeneration from Injury in the Olfactory Organ of Adult Spiny Lobsters**

Support for our hypothesis comes from the positive correlation between *splash* expression and ablation-induced proliferation, suggesting a role for *splash* in regeneration of a damaged or ablated olfactory organ. Our data show that ablation of the distal end of the LF, which enhances the rate of cell proliferation resulting in regeneration (Harrison et al., 2003, 2004), also enhances the level of *splash* labeling in the LF proliferation zone (Fig. 7). We observed a dramatic increase of *splash* labeling in epithelial cells and ORN precursor clusters in the proliferation zone at 7 and 9 days after ablation (Fig. 7B, D) compared with 5 days post-ablation (Fig. 7A, C).

Another animal model that is instructive to our understanding of neurogenesis associated with regeneration is limb regeneration in fiddler crabs. An expressed sequence tag library of the blastema four days after limb removal, and thus early in regeneration, failed to identify any *ASC* homologs but did identify an *enhancer of split* homolog (Durica et al., 2006). Interestingly, the spiny lobster olfactory organ also expresses a *hairy - enhancer of split* homolog (Chien et al., 2009), though its role in regeneration has yet to be examined. In lobsters, damage to or ablation of the LF also enhanced proliferation zone enriched transcript (*PET-15*), which is thought to be a regulator of endogenous proteases in proliferating epithelia, in PZ (Stoss et al., 2004). Thus, we believe that the expression of two genes with possibly different functions, *splash* and *PET-15*, is regulated by diffusible signals from damaged tissue.

Regeneration of ORNs shares many similarities but also some differences with normal neurogenesis in the context of changes in gene expression (Gordon et al., 1995; Manglapus et al., 2004; Shetty et al., 2005). Our study demonstrates that *splash* is one of the genes that is not involved in normal ORN formation but is involved in regeneration (Fig. 7B, D). A recent study of zebrafish showed that *sox2* transcription factor is required for regeneration but not during initial development of hair cells (Millimaki et al., 2010). It would be interesting to determine whether inhibiting *splash* expression inhibits regeneration.

Multiple Roles of *splash* in Lobster Olfactory Organ

We propose that *splash* has multiple roles, based on its expression in a diversity of cell types in different parts of the LF and over different molt stages. *splash* is expressed in epithelial cells located in the ventral and dorsal PZ and MZ, in auxiliary cells, in immature and mature ORNs associated with aesthetasc sensilla, and in large bipolar sensory neurons innervating

non-aesthetasc sensilla. *splash* is not expressed in some other cells types, including granulocytes and chromatophores. Thus, *splash* expression in the LF is not exclusively in tissue involved in the formation of ORNs, and it is consistently expressed in mature sensory neurons of aesthetasc and non-aesthetasc sensilla. To our knowledge, *ASC* expression and function in adult arthropods have not been examined. Therefore this is the first demonstration of expression of an *ASC* homolog in mature neurons in arthropods.

There are multiple functional possibilities for *splash* expression in multiple cell types. Firstly, *splash* may function as a transcription factor whose primary role is in the maintenance of sensory neurons. For example, *NeuroAB*, a novel bHLH transcription factor, is expressed in mitotic cells; however, its predominate expression in bipolar and amacrine cells in the developing and mature chick retina suggests that it functions in the maintenance of neurons rather than in proliferation (Ohkawara et al., 2004). *sox2* encodes a transcription factor involved in maintenance of stem cells (Avilion et al., 2003) and formation of sensory epithelium in the inner ear of mice (Hume et al., 2007) and chicks (Neves et al., 2007), but it has other roles in developing zebrafish. Although progenitors of hair cells and support cells express *sox2*, knockdown studies showed no effect on hair cell formation but an effect on maintenance of hair cells and support cells (Millimaki et al., 2010). In *Drosophila*, expression of *engrailed* (Blagburn, 2008) in mature olfactory sensory neurons suggests additional functions in maintenance rather than in early development. Taken together, transcription factors previously known for functions during early development are now known to have additional roles in later stages, and *splash* may be another example.

Secondly, *splash* may have dual functions: it may have transcriptional and non-transcriptional roles depending on the developmental stage (immature vs. mature cells, or larvae vs. adult) or cell type in which it is expressed. Eyes absent (*Eya*) has two functions: as a transcription factor and a tyrosine phosphatase during eye development (Tootell et al., 2003; Rebay et al., 2005). Other bHLH transcription factors have intercellular functions rather than as the more typical intracellular signals (Prochiantz and Joliot, 2003). *splash* maybe a transcription factor but have anti-proneural rather than proneural functions as shown for Daughterless (*Da*) in eye development of *Drosophila* (Lim et al., 2008).

Thirdly, *splash* may function as a transcription factor but regulate the expression of genes that regulate the physiology but not the development of cells. In adult *C. elegans*, within a few hours after altering the expression of *lin-12* Notch receptor homolog using inducible RNAi and conditional gain of function, changes in locomotory behavior were observed suggesting an effect on the physiological properties of neurons and not in cell fate specification (Chao et al., 2005). *lin-12* genetically interacts with an AMPA/kainate receptor homolog *glr-1* expressed in command interneurons suggesting a physiological mechanism for a developmental gene. The novel finding that *lin-12* functions in the neuronal physiology via the canonical transcriptional activation in adults is further evidence that there may be more transcription factors that have developmental and additional unidentified functions. It is likely that similar to *lin-12*, *splash* may function in mature ORNs or bipolar cells by regulating the expression of various ion channels. In summary, although our results do not demonstrate *splash* as a transcription factor or a non-transcriptional function, based on it being an *ASC* homolog, it is likely that one of its function is as a transcription factor. Our data suggest that *splash* may be another example of transcription factors having multiple functions, although more work is necessary to elucidate the nature of these functions.

Expression of an Ionotropic Glutamate Receptor Specific to Mature ORNs of Spiny Lobsters

Our need for an ORN-specific label for *in situ* hybridization led us to clone a spiny lobster homolog of an ionotropic glutamate receptor subunit, *GluR1*. This was based on previous

work on the clawed lobster *H. americanus* which identified a *GluR1* homolog (OET07) that is specifically expressed in all aesthetasc ORNs and not in other cell types (Hollins et al., 2003; Stepanyan et al., 2004, 2006). Our partial sequence of *P. argus GluR1* is highly similar to its *H. americanus* homolog and *P. argus GluR1* is also expressed in all ORNs. The function of *GluR1* is unknown. However, Corey et al. (2010) proposed that the olfactory organ of spiny lobsters has additional ionotropic receptors besides *GluR1* and that they have sequence similarity to ionotropic receptors expressed in antennal olfactory neurons in *Drosophila* (Benton et al., 2009). This raises the intriguing hypothesis that the lobster's ionotropic receptors might be involved in olfactory transduction, and that *GluR1*, since it is expressed in all ORNs, is a co-receptor functionally related to the insect Or83b.

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LIST OF ABBREVIATIONS

AE	aesthetasc sensilla
ASC	achaete-scute complex
ATG	aesthetasc tegumental glands
bHLH	basic-helix-loop-helix
BrdU	5-bromo-2'-deoxyuridine
D-Ep	dorsal epithelium
dPZ	distal proliferation zone
GluR1	ionotropic glutamate receptor
ICC	immunocytochemistry
ISH	<i>in situ</i> hybridization
LF	lateral flagellum
mPZ	middle proliferation zone
MZ	mature zone
NAE	non-aesthetasc sensilla
ORN	olfactory receptor neuron
PA-5/7/9	post-ablation days 5/7/9
pH3	phosphorylated histone 3
pPZ	proximal proliferation zone
PZ	proliferation zone
splash	<i>spiny lobster achaete-scute homolog</i>
V-Ep	ventral epithelium

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Figure 1.

Schematic drawings of the antennular lateral flagellum (olfactory organ) of the adult spiny lobster *P. argus*. **A** A pair of antennules (A1: first antennae) is located on the head of the animal. Each antennule has two flagella, a medial (MF) and a lateral (LF). **B** Each flagellum is comprised of repeated cuticular units called annuli (AN). Each annulus in the distal tuft (T) region of the LF bears ca. 20 olfactory sensilla called aesthetascs (AE). Non-aesthetasc sensilla (NAE) are located on mesial (Ms), lateral (L), dorsal (D), and ventral (V) sides of both flagella. **C** Sagittal section of LF showing aesthetasc ultrastructure. Each aesthetasc is innervated by the outer dendrites of about 300 bipolar olfactory receptor neurons (ORNs). The inner dendrites are surrounded by auxiliary cells. ORN axons project into the brain. **D** LF developmental zones are divided into regions of 6 annuli with proximal to the left and distal to the right. Throughout the animal's life, aesthetasc sensilla and their associated ORNs are added to the LF in the proliferation zone (PZ), mature and become chemoresponsive in the mature zone (MZ), and are then shed in the senescence zone (SZ). In this figure, each annulus is given a number, with '1' representing the most proximal aesthetasc-bearing annulus, annuli proximal to '1' having progressively larger negative numbers, and annuli distal to '1' having progressively larger positive numbers. According to this numbering scheme, the PZ is represented by annuli -10 to 12, with the proximal PZ being annuli -10 to -5, the middle PZ being annuli -4 to 4, and the distal PZ being annuli 5 to 12. The mature zone is represented by annuli 13 to 52, and the senescence zone is

represented by annuli 53 and greater. Drawings are modified from Grünert and Ache (1998), Harrison et al. (2004), and Schmidt et al. (2006)

Figure 2.

In situ hybridization with digoxigenin RNA probes for *splash* in the antennular lateral flagellum of an adult intermolt spiny lobster. **A** Cellular morphology of the tuft region of the lateral flagellum (LF) in an unlabeled sagittal section. All subsequent sections are presented in this orientation. The epithelium on the ventral surface of the LF (V- Ep) is indicated by a bracket. Two of the four olfactory receptor neuron (ORN) clusters shown in this image are outlined in black. **B–G** Results using *splash* RNA open reading frame (ORF) probe or *splash* RNA bHLH 5' probe in the proliferation zone. **B** Ventral epithelium (arrow) in proximal PZ, which lacked ORN clusters, was uniformly labeled by *splash* ORF probe. **C, D** Newly formed ORN clusters in the middle PZ were intensely and abundantly labeled by *splash* ORF probe (asterisks), although some ORN clusters were completely devoid of labeling (indicated by outline in **C**), and some cells within an ORN cluster were also devoid of labeling (arrows in **D**). **E** *splash* bHLH 5' probe labeling of ORN clusters (asterisk) was qualitatively similar but less intense compared to *splash* ORF probe, including some cells within a labeled ORN cluster were not labeled (arrow). **F** Multipolar chromatophores (arrows) and **G** granulocytes (arrows) did not label with either probe. **H–J** Positive control using RNA probes for an ionotropic glutamate receptor subunit (GluR1). **H** Antisense RNA probe for GluR1 labeled the majority of ORNs in the mature zone (asterisks). **I** Sense RNA probe for GluR1 showed no labeling of ORNs (outline) in the mature zone. **J** Double labeling using anti-GluR1 antibody (red) and the nuclear marker Hoechst 33258 (blue) showed labeling of ORN somata (asterisks) and inner dendrites (arrow) in the mature zone. **K–M** Negative controls for digoxigenin (DIG) labeled RNA *in situ* hybridization with NBT/BCIP (purple) using immature and mature zones. **K** Control using hybridization buffer without *splash* RNA probe. **L** Control treated with RNase A prior to the application of *splash* RNA probes. **M** Control lacking primary antibody. All three negative controls showed no *splash* specific labeling, but there was non-specific labeling of the cuticle (arrow) and aesthetasc tegumental glands (data not shown). Scale Bars 50 μ m (**A–D**), 20 μ m (**E–G**), 50 μ m (**H–I**), 20 μ m (**J–M**)

Figure 3.

Effect of molt stage on *splash* labeling. **A** Cellular morphology in the mature zone of an unlabeled antennular lateral flagellum, showing the ventral (V-Ep) and dorsal (D-Ep) epithelium in sagittal section. **B–C** Confocal micrographs showing proliferating cells in the proliferation zone (PZ) of an intermolt animal. **B** BrdU labeling (after a single injection of BrdU and 24-h survival) together with Hoechst 33258 staining showing BrdU+ (S-phase) cells (red) in a newly formed ORN cluster proximal to the V-Ep. BrdU+ cells had diverse nuclear shapes (arrows). **C** Triple labeling with anti-BrdU (red), anti-phosphorylated histone 3 (pH3) (green), and Hoechst 33258 (blue), showing a pH3+ (M-phase) cell (arrow) in an ORN cluster containing BrdU+ (S-phase) cells. **D–G** *splash* bHLH 5' probe labeling in a lateral flagellum of an intermolt animal. **D** Proximal proliferation zone (PZ), which lacked ORN clusters, had intense *splash* labeling in most of the V-Ep (arrow). **E** Middle PZ had *splash* labeling in epithelial cells and in most cells of newly formed ORN clusters (asterisks). **F** Distal PZ had intense *splash* labeling in the V-Ep, and very intense labeling in many ORN somata (asterisks), and in auxiliary cells surrounding the ORN inner dendrites (white arrow). Each ORN cluster contained some unlabeled cells. Aesthetasc tegumental glands (ATGs), which are abundant in the aesthetasc bearing region of the LF, were non-specifically labeled (see also Fig. 2). **G** Mature zone had very intense *splash* labeling including *splash*+ cells in the ORN clusters (asterisks). **H**: Determination of molt stage based on pleopod morphology showed that this intermolt animal did not have new setae beneath the cuticle. **I–L**: *splash* 5' bHLH probe labeling in the lateral flagellum of a premolt animal. Proximal PZ contained newly formed aesthetasc sensillum (black arrow) that will emerge on the LF cuticular surface after the animal's next molt. Auxiliary cells (white arrow) and some cells in ORN clusters (asterisk) had intense *splash* labeling, but some ORN clusters were unlabeled. **J**: Distal PZ had intense *splash* labeling in the V-Ep. The inner dendrites were surrounded by intensely labeled auxiliary cells (arrow). Most ORN clusters (asterisks) contained both *splash*+ and *splash*– cells. **K** Mature zone was similar in *splash* labeling in V-Ep compared to middle PZ. There were many ORNs in MZ with intense labeling (asterisk). **L** Premolt animal had new cuticle with new setae (arrowhead) beneath the old cuticle (arrow) that will shed at the next molt. **M–N** Dorsal epithelial cells (D-Ep) in proximal PZ (**M**) showed very intense *splash* labeling in the cytoplasm compared to MZ (**N**). *splash* labeling in D-Ep of PZ is more intense than in V-Ep. Scale Bars 100 μ m (**A**), 10 μ m (**B**), 20 μ m (**C**), 50 μ m (**D–J**), 20 μ m (**K–N**)

Figure 4.

The mean percentage of *splash+* and *splash-* cells in the antennular lateral flagellum. Data are shown for the proliferation zone (PZ) and mature zone (MZ) of premolt animals (left figure) and intermolt animals (right figure). In the PZ, data are shown for ORN precursor cells and ORN clusters; in the MZ, data are shown for ORNs. Values are mean $\pm$ SEM for 3 animals per molt stage. The majority of cells in both regions (PZ, MZ) and for both molt stages (premolt, intermolt) were *splash+*. The only molt-stage difference was that compared to premolt animals, intermolt animals had an even higher percentage of *splash+* ORNs in the MZ.

Figure 5.

splash and BrdU labeling in intermolt proliferation zone (PZ) of the antennular lateral flagellum. **A** After a single injection of BrdU followed by a 24-h survival period, numerous BrdU+ cells were located in the ventral epithelium (white arrows) and other areas (white arrowheads). Double labeling is with the nuclear marker, Sytox Green. **B** Confocal scan of *splash* RNA labeling (black) and anti-BrdU labeling (red) in the PZ of a premolt animal showed one BrdU+/*splash*+ cell (i) and one BrdU+/*splash*- cell (ii) in the ventral epithelium. Isolated BrdU+ cells (arrowheads) were BrdU+/*splash*-. **C** Two adjacent cells in the ventral epithelium were either BrdU+/*splash*+ (asterisk) or BrdU-/*splash*+ (black arrow). **D** The mean $\pm$ SEM values of the percentage of BrdU+/*splash*+ (gray bars) and BrdU+ cells (white bars) in the PZ. Only a small percentage of BrdU+ cells were labeled with *splash* in both the ventral epithelium (6%, $n = 15$) and newly formed ORN clusters (18%, $n = 58$), compared with cells labeled with BrdU only (94 %, $n = 126$ and 82%, $n = 230$) (number of animals = 3). *Scale Bars* 20 μ m (**A**), 50 μ m (**B**), 10 μ m (**B i, ii**), 10 μ m (**C**)

Figure 6. *splash* labeling of non-aesthetasc sensory neurons in the antennular lateral flagellum. **A–H** Large somata of non-aesthetasc sensory neurons with typical bipolar morphology were *splash*+. Some had *splash* labeling in the nucleolus (white arrows) (**A**, **D**, **F**). These cells occurred in singlets (**C**, **D**), pairs (**A**, **B**), or groups of 4–6 (**E–H**) in the lateral, mesial, dorsal, and occasionally ventral regions of the LF. **I–L** Confocal images of immunostained sections of LF showing large bipolar cells sensory neurons. **I** Bipolar cells (asterisks) with thick dendrites (white arrows) innervating non-aesthetasc sensilla were labeled with anti-acetylated tubulin (red) and stained with Hoechst 33258 (blue). **J** Other types of bipolar cells (white arrows) double labeled with anti-acetylated tubulin (red) and Hoechst 33258 (blue) were located dorsally. **K** Large bipolar cells stained with nuclear marker Hoechst 33258 (blue) had a distinctive nuclear morphology and did not label with anti-GluR1 (green), unlike ORNs (**L**). Scale Bars 20 μ m

Figure 7.

Ablation-induced *splash* labeling in antennular lateral flagella of intermolt animals. Schematic drawing showing unilaterally-ablated lateral flagellum and areas examined at post-ablation days 5 and 9 (PA-5 and PA-9 respectively). **A, B:** Proximal PZ. **C, D:** Middle PZ. **E–F:** Distal PZ. **G–I:** Mature zone. **A** The proximal PZ at PA-5 showed faint intensity *splash* labeling in some ventral epithelial cells. The ORN clusters were not *splash*+. **B** The proximal PZ at PA-9 showed very intense *splash* labeling in the ventral epithelial cells and in numerous ORN clusters (asterisk). **C** Middle PZ at PA-5 did not show *splash* labeling in ventral epithelium (V-Ep) (arrow). **D** PA-9 V-Ep in middle region appeared to detach from the cuticle. Most if not all epithelial cells and ORN clusters (asterisks) had very intense

splash labeling compared to PA-5. **E** ORN clusters in PA-5 distal PZ were labeled with *splash* (asterisks). There were many unlabeled ORNs within clusters containing labeled ORNs. **F** Distal PZ PA-9 showed very intense *splash* labeling in most of the epithelial cells and ORNs (asterisk). Similar to PA-5, some ORNs were unlabeled. **G** At PA-5, the epithelium most proximal to the blastema had intense *splash* labeling. **H** At PA-9 and most proximal to the blastema, there were many epithelial cells with very intense *splash* labeling. **I** Lateral and mesial epithelial cells proximal to the blastema contained the most intense labeling and the greatest number of cells labeled with *splash* at PA-9 compared to PA-5. *Scale Bars* 50 μm (**A–D**), 20 μm (**E, F**), 20 μm (**G–I**)

Table 1

Median intensity rating for *splash* labeling in premolt and intermolt LF. Symbols are as follows: not examined (*), no labeling (-), faint labeling (+), intense labeling (++), very intense labeling (+++). In intermolt animals, epithelial cells in the proximal, middle, and distal proliferation zone (pPZ, mPZ, dPZ) were intensely labeled with *splash*, and epithelial cells and ORNs in the mature zone (MZ) had very intense *splash* labeling. In premolt animals, epithelial cells in the pPZ, mPZ, dPZ, and MZ had intense *splash* labeling, and ORNs in the dPZ had faint *splash* labeling.

	Premolt (N = 3)		Intermolt (N = 3–5)	
	Epithelium	ORNs	Epithelium	ORNs
pPZ	++	*	++	*
mPZ	++	*	++	*
dPZ	++	+	++	+++
MZ	++	++	+++	+++

Table 2

Median intensity rating for *splash* labeling in ablated antennular lateral flagella of intermolt animals. Symbols are as follows: not examined (*), no labeling (–), faint labeling (+), intense labeling (++), very intense labeling (+++). Post-ablation days 5 and 9 (PA-5 and PA-9 respectively). Epithelial cells showed faint intensity of *splash* labeling in PA-5 proximal proliferation zone (pPZ) and no *splash* labeling in ORN precursors. PA-9 epithelial cells and ORN precursors in pPZ and ORNs in dPZ had very intense *splash* labeling. Epithelial cells in the site of injury of PA-9 animals had very intense *splash* labeling, compared with PA-5 animals which had intense labeling.

	P5 (N = 3)		P9 (N = 3)	
	Epithelium	ORNs/ precursor	Epithelium	ORNs/ precursor
pPZ	+	–	+++	+++
dPZ	++	++	+++	+++
Site of Injury	++	*	+++	*