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Development of Mechanosensory Innervation in the Frog, Xenopus Laevis

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DEVELOPMENT OF MECHANOSENSORY INNERVATION IN THE FROG, *XENOPUS LAEVIS*

by

Peter Feuk

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ABSTRACT

DEVELOPMENT OF MECHANOSENSORY INNERVATION IN THE FROG, *XENOPUS LAEVIS*

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The University of Wisconsin-Milwaukee, 2017
Under the Supervision of Dr. R. David Heathcote

This study aims to investigate whether a specific target cell in the epidermis of the African clawed frog, *Xenopus laevis,* guides the initial outgrowth and pattern of Rohon-Beard (RB) cells and their survival. RB cells are primary mechanosensory neurons present during the early developmental stages of *X. laevis.* These neurons provide sensory input to the frog throughout embryonic and larval development before initiating apoptosis around the start of metamorphosis. The innervation of embryonic skin cells by RB neurons exhibits a distinct pattern that features encircling of a specific subset of epidermal cells. We hypothesize that encircled cells could be a recently discovered cell type that synthesizes and secretes serotonin. To test whether these small secretory cells (SSC) play a chemoattractive role in the innervation of primary mechanosensory neurons in *X. laevis,* we used a variety of approaches. By immunolabeling RB processes in the epidermis, we established the wild-type pattern and quantity of innervation. Gain of function and loss of function tests utilizing pharmacological modulators of serotonin, directly examined the role of SSCs. We showed that serotonin levels can be over or under expressed, and that

there is a quantifiable response of the resulting mechanosensory innervation. RB neurons innervating larval skin lacking serotonin showed a lower frequency of encircled SSCs as well as a greater number of retraction bulbs. We also showed a correlation between behavior and serotonin levels in the epidermis. Loss of function treatments showed a higher proportion of animals failing to respond to stimuli and an increase in inappropriate escape responses. Finally, we related changes in *X. laevis* larval skin and mechanosensory neurons during metamorphosis to tissue remodeling and the transition to adult sensory function. This research provides insight into axonal guidance and the patterning of mechanosensory innervation in *X. laevis.* It stands as an example of how the innervation of the vertebrate skin is established and maintained between developmental stages.

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ACKNOWLEDGEMENTS

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Last, I would like to thank my family. I'm grateful my parents supported and encouraged me in many different ways during my studies. They taught me to always work hard towards my goals and to strive for success in everything. Finally, I'd like to thank my wife, Ashley. She has been nothing but helpful throughout this entire degree. Always there to bounce ideas off of, practice a presentation in front of, or just show her support when needed.

Introduction

Understanding how the nervous system operates is a fundamental issue for all neuroscientists. In order to understand how the nervous system works, it is important to study how it is put together during early stages of development. To that end, we have investigated the emergence and subsequent innervation of an early developing mechanosensory system in the frog, *Xenopus laevis*. Mechanosensation is vital to this and many other animals during their early stages of development. It plays an important role in mediating the hatching response, as well as providing initial input from the environment after hatching. The primary mechanosensory neurons differentiate during embryogenesis and gradually innervate their targets in the skin.

In the African clawed frog, *Xenopus laevis*, and many other vertebrates, Rohon-Beard (RB) neurons are the first or primary neurons that mediate mechanosensation during embryonic and larval development. These neurons were independently discovered by Rohon (1885) and Beard (1889; 1892; 1896) in the neural tube of lower vertebrates. Their large size and distinct location have made RB neurons an important model for neuron development and patterning. They originate from the lateral edges of the developing neural plate, and are found dorsally in the neural tube after neural fold closure (Chitnis et al. 1995). Once differentiated and established in the neural tube, RB neurons send large growth cones laterally and then ventrally to innervate the embryonic epidermis. In *X. laevis*, RB neurons start undergoing apoptosis at the beginning of metamorphosis (Lamborghini, 1987) and their sensory function is taken over by dorsal root ganglia (DRG) in the adult

frog. The study of RB mechanosensory neurons has provided extensive information about growth cones, axon guidance, as well as the embryonic development of sensory cells.

The patterning of innervation by RB neurons develops initially during embryonic stages when axons are sent to the periphery along the surface of the myotomes (Taylor and Roberts, 1983). RB growth cones then enter the basal lamina where they grow to and contact the basal surface of superficial epidermal cells. At this stage scattered epidermal cells are completely encircled by RB neurites. Other skin cell types are partially innervated by RB processes, but a specific subset are entirely surrounded (Somasekhar and Nordlander, 1997). We believe this subset of cells is a recently described class of epidermal cells, named small secretory cells (SSCs; Walentek et al. 2014).

SSCs are characterized by their conical shape, pattern of distribution, and the synthesis and secretion of serotonin in large granular vesicles. SSCs were characterized by Walentek et al. (2014) while establishing *X. laevis* skin as a model experimental system for mucociliary epithelium (MCE) that is similar to human airway epithelium. *X. laevis* larval skin, like MCE, has specialized mucus secreting goblet cells and multi-ciliated cells (MCCs) that provide directional transport of mucus. The investigators present evidence for SSCs regulating the ciliary beating of MCCs, therefore contributing to the transport of mucus across the skin. They show that the elimination of SSCs within larval epidermis contribute to a decrease in velocity of ciliary-driven mucus across the skin epithelium.

Our research into SSCs is based upon the assumption that they are the subset of *X. laevis* skin cells that are completely encircled by RB mechanosensory neurons. Serotonin is first labeled in *X. laevis* skin around the same time that RB cell bodies send growth cones to

innervate the epidermis (Walentek et al. 2014). Our hypothesis is that SSCs have another role in the larval epidermis; they are chemoattractive to RB growth cones and they contribute to the pattern and maintain the mechanosensory plexus in larval *X. laevis* skin.

Our experiments establish a wild-type pattern of innervation of primary mechanosensory RB neurons as well as the distribution of SSCs in the larval skin. We then analyzed whether SSCs and RB neurites are co-localized at early tail bud stages in *X. laevis.* Following these investigations, we utilized pharmacological manipulation of serotonin present in the epidermis to analyze the difference in innervation of skin cells in the presence and absence of serotonin. We also performed behavioral escape experiments to see if there was a functional consequence in larval tadpoles lacking serotonin in their skin. Finally, we examined stages where tissue remodeling should occur to test whether the fate of SSCs is related to the apoptosis of RB mechanosensory neurons. These experiments provide insight into axonal guidance and also patterning mechanisms underlying mechanosensory innervation in *X. laevis* skin. They also provide a model for the innervation and patterning of vertebrate skin during its development.

Materials and Methods

Xenopus laevis embryos and larvae were obtained from the breeding colony maintained in our laboratory. The times and stages of development at 23˚C were determined according to the criteria of Nieuwkoop and Faber (1994). Bath application of pharmacological agents was done between 1.0 (st 22) and 1.85 (st 33/34) days of development (Fig. 1). During this period, RB axons grow to the skin and serotonin is expressed there. At 4.4 days (st 46), the animals start metamorphosis and RB neurons begin to die.

HNK-1 Labeling of Mechanosensory Neurons

Animals were anesthetized in 1 mM Tricaine and then fixed before visualizing the sensory neuron processes. Immunocytochemical marking of the HNK-1 antigen, which is expressed on the surface of Rohon Beard neurons (Nordlander, 1989, 1993; Metcalfe et al. 1990), involved fixation in 4% paraformaldehyde (PFA, pH 7.4) at 4˚C for 1 hour. After washing in 0.1 M Phosphate buffered saline (PBS), and dehydration in methanol, the embryos were bleached in 1.74 % H_2O_2 to remove pigment. Following rehydration, non-specific binding was blocked with PBT (0.1 M PBS, 0.2% Bovine serum albumin (BSA) and 0.1% Triton X-100). The embryos were incubated overnight at 4˚C in mouse anti HNK-1 monoclonal antibody (Miles Epstein, UW Madison, C0678 from Sigma Aldrich) diluted 1:250 in PBT. After incubation with HNK-1, the embryos were washed in PBS three times, for thirty minutes each, and incubated in biotinylated horse anti-mouse secondary antibody (Vector Laboratories) for 1 hour at room temperature, diluted 1:200 in PBT. After

washing, the embryos were incubated in avidin-biotin horseradish peroxidase (HRP) complex (ABC Elite, Vector Laboratories) for 1 hour at room temperature, diluted 1:50 in PBT. Diaminobenzadine (DAB, 1 mg/ml in PBT) intensified with $Co(Cl_2)_2$ and $Ni(NH_4)_2(SO_4)_2$ was catalyzed by H_2O_2 and used to visualize the HRP complex. The embryos were washed, infiltrated in 90% glycerol in PBS, whole-mounted, and viewed with a Zeiss Axioskop using brightfield and Differential Interference Contrast (DIC) optics.

Serotonin Labeling of SCCs

Immunocytochemistry for serotonin (5-hydroxytryptamine), a monoamine neurotransmitter derived from tryptophan (Gershon et al. 1965) was used to mark the serotonergic vesicles contained in small secretory cells (SSCs) found in *X. laevis* larval epidermis. The same protocol as HNK-1 immunocytochemistry was utilized except the primary antibody was a rabbit polyclonal to serotonin (S5545 from Sigma Aldrich) diluted 1:250.

Double Immunofluorescent Labeling

Double immunolabeling of HNK-1 and serotonin was performed to confirm co-localization in larval *X. laevis* skin. The same protocol was used for both HNK-1 and serotonin immunocytochemistry by incubating in both primary antibodies. Following this, animals were incubated in fluorescent secondary antibodies; Alexa Fluor 568 (red) goat

anti-mouse to detect HNK-1 and Alexa Fluor 488 (green) goat anti-rabbit to detect serotonin. Animals were then examined on a confocal microscope (Leica SP2).

Gain and Loss of Function Experiments

Bath application of pharmacological compounds 5-hydroxytryptophan (5-HTP) and 4-Chloro-DL-phenylalanine methyl ester hydrochloride (PCPA) provided gain and loss of function experiments respectively. Animals were anesthetized in 1mM Tricaine and fixed in 4% paraformaldehyde (PFA, pH 7.4) at 4˚C for 1 hour, then incubated in either 10μm 5-HTP or 80μm PCPA for 24 hours. The same protocol for immunocytochemistry was then utilized for labeling animals with either HNK-1 or serotonin.

Experiments on Behavioral Effects

Animals were analyzed for behavioral changes following bath application of pharmacological modulators 5-HTP and PCPA. Soon after hatching (1.85dpf), tadpoles were incubated in 0.1X Modified Barth's Solution (MBS), 5-HTP, or PCPA overnight. Trunk regions were then stroked with an ultra-fine flexible glass filament and escape responses were classified according to methods utilized previously (Boothby and Roberts, 1993). Behaviors recorded included what direction the animal escaped from the stimulus (lateral vs. contralateral) as well as what type of escape was witnessed. These included V-flexions (bending only at the middle of the trunk), C-flexions (widespread bending forming a C

shape), or simply swimming away from the stimulus. Statistical analysis of the effect of treatments utilized one-way ANOVA with Tukey-Kramer multiple comparison post-test.

Visualization of Programmed Cell Death

To label cells actively undergoing apoptosis, we utilized the terminal deoxynucleotidyl transferase dUTP nick end-labeling (TUNEL) assay using the Apoptag kit (S7110 from EMD Millipore). Animals were fixed in 4% PFA, pH 7.4, overnight at 4˚C. After washing in 0.1 M PBS, and dehydration in methanol, the embryos were bleached in 1.74 % H_2O_2 to clear pigment. Following rehydration, non-specific binding was blocked with PBT. Incubation in equilibration buffer on a shaker at room temperature for 1 hour followed, then overnight incubation in terminal deoxynucleotidyl transferase (TdT) enzyme solution (77µl reaction buffer + 33µl TdT enzyme) on a shaker at room temperature. Specimens were then incubated at 37˚C for 1 hour, TdT enzyme solution removed, and incubated in stop buffer for 1 hour at room temperature. They were then washed in maleic acid buffer (MAB, 100mM maleic acid, 150mM NaCl, 0.1% Tween-20, 7.9g/L NaOH, pH 7.5) for 10 minutes and incubated in blocking solution for 1 hour at room temperature. Anti-digoxigenin fluorescein isothiocyanate (green) in blocking solution (68µl blocker + 62µl anti-DIG) was then applied overnight on a shaker at 4˚C followed by a 10 minute rinse in MAB, 1 hour incubation at room temperature in propidium iodide (1:1000 in MAB), a wash in PBT, and mounting in 90% glycerol.

Analysis

Quantification of Vesicles

Following fixation, immunocytochemistry, or immunolabeling, animals were visualized and traced using a *camera lucida* attachment on a Zeiss Axioskop using brightfield and DIC optics. Quantification of serotonin vesicles was done by sampling three 40,000μm^2 areas in the larval skin and averaging the number of vesicles, cells, and vesicles per cell in different regions of the animal. Statistical analysis of the effect of treatments used one-way ANOVA with Tukey-Kramer multiple comparison post-test. Dorsal regions were grouped together as were ventral.

Quantification of Neurites

Quantification of mechanosensory axons was performed using a digitizing tablet (Drawingboard IV) and analysis software (Sigma ScanPro). Sampling was similar to serotonin quantification, but used three 10,000μm^2 areas to determine average axon length, number of retracted axons/bulbs, and number of encircled cells. Statistical analysis was the same as for serotonin treatments.

Figure 1

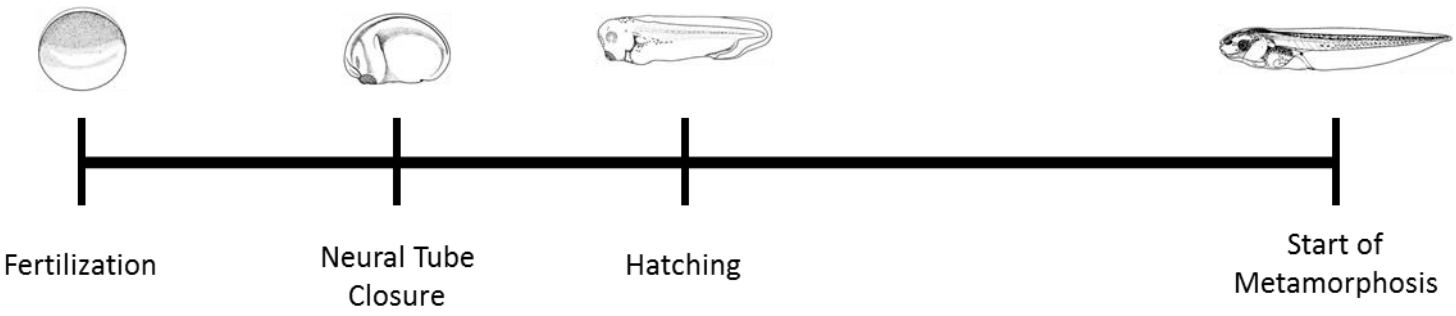

Figure 1: Timeline of *X. laevis* developmental stages. Fertilization - st. 1, 0dpf. Neural Tube Closure - st. 22, 1.0dpf. RB neurons begin to grow to the periphery and production of serotonin starts in the skin. Bath application of pharmacological agents were initiated at this stage of development. Hatching - st. 33/34, 1.85dpf. RB innervation pattern is established and large serotonin vesicles are present in the skin. Start of Metamorphosis - st. 46, 4.42dpf. Serotonin vesicles are no longer in the skin and RB neurons start undergoing apoptosis.

Results

Serotonin and sensory axons in the skin

Establishing the wild type innervation and patterns

To address whether SSCs in the skin play a chemoattractive role in mechanosensory innervation, we first established the wild-type pattern of skin cells containing serotonin and the pattern of RB neurites innervating larval skin. We selected early tailbud developmental stages (Nieuwkoop and Faber, 1994) for our experiments, as this is when SSCs first appeared in the larval skin and coincided with extensive RB outgrowth and innervation. *Camera Lucida* tracings establish the wild-type distribution and patterning for both serotonergic vesicles and HNK-1 labeled mechanosensory axons (Fig. 2). In wild-type animals, serotonin occurs in small uniform clusters of vesicles throughout the skin (Fig. 2A). The bulk of wild type serotonin labeling is over the trunk muscles, but a small portion of vesicles are in the dorsal fin region. HNK-1 labeled sensory axons are also distributed evenly and feature small encircled regions around 10μm in diameter throughout the skin (Fig. 2B). We hypothesize that encircling occurs around SSCs that contain serotonin vesicles.

Effects of increasing serotonin on sensory axons

To test our hypothesis that SSCs are the encircled cells in *Xenopus* larval epidermis, we increased the amount of serotonin in the skin. 5-hydroxytryptophan (5-HTP) is the immediate precursor to serotonin in the serotonergic synthesis pathway. By incubating

larval tadpoles in 5-HTP, the additional precursor resulted in increased serotonin (Udenfriend et al. 1956). We hypothesized embryos incubated in 5-HTP would show an increase in vesicles in SSCs located in the skin and more cells encircled by RB processes. *Camera Lucida* tracings of animals treated with 5-HTP show a larger number of serotonergic vesicles and a higher density of vesicles throughout the larval epidermis than controls (Fig. 3A). Vesicles also appear more numerous in the dorsal fin area of the animal. After treatment with 5-HTP, neurites labeled with HNK-1 also show more cells encircled by axons (Fig. 3B). This is consistent with serotonin in the SSCs serving as a chemoattractant for sensory axons.

Effect of decreasing serotonin on sensory axons

To further examine our hypothesis that SSC's are the encircled cells in *Xenopus* larval epidermis, we decreased the amount of serotonin in the skin. We used 4-Chloro-DL-phenylalanine methyl ester hydrochloride (PCPA) to inhibit the rate-limiting enzyme (tryptophan hydroxylase) in the conversion of 5-HTP to serotonin which reduces the amount of serotonin present (Koe and Weissman, 1967). We expected to see few or no serotonergic vesicles in the larval skin along with fewer RB mechanosensory neurites encircling cells. *Camera Lucida* tracings of animals treated with PCPA showed no serotonergic vesicles present compared to age-matched controls (not shown). Tracings of HNK-1 labeled mechanosensory axons display a clear aberrant configuration of innervation, a lower frequency of encircling, as well as a large number of neurites with

retraction bulbs after incubation in PCPA (Fig. 4). This is also consistent with serotonin in the SSCs serving as a chemoattractant for sensory neurons.

Co-localization of serotonergic vesicles and sensory axons

In order to test whether serotonin vesicles were surrounded by mechanosensory axons we performed double immunofluorescent labeling. We hypothesized that SSCs would be encircled by RB mechanosensory neurites. Epifluorescent images from 1.85dpf animals show that serotonin containing cells are surrounded by HNK-1 labeled sensory axons (Fig. 5). Serotonergic vesicles are mostly found near the apical surface of SSCs indicating the serotonergic vesicles are ready to be secreted at this time. These results are consistent with the hypothesis that sensory axons encircle SSCs as they grow into the skin.

Quantification of sensory axons and their potential targets

Serotonergic vesicle quantification

In order to estimate the number of serotonergic vesicles following manipulation of serotonin, we counted vesicles in dorsal and ventral regions of the developing animal. These regions were separated after one-way ANOVA statistical analysis showed them to be significantly different. Visual examination of the *camera lucida* tracings, led to the hypothesis that there would be more serotonergic vesicles present in 5-HTP treatments compared to controls and less present in PCPA treatments compared to controls. Quantification of vesicles shows a significant ($p < 0.05$) increase in vesicle number over

control in both dorsal and ventral regions following 5-HTP treatment. Furthermore, PCPA treatments eliminate serotonergic vesicles completely in each region (Fig. 6). Although the difference with controls is not significant in dorsal regions, it is significantly different in the ventral regions and is significantly different from 5-HTP treatments in both regions. The pharmacological treatments clearly lead to over and under expression of serotonergic vesicles in the skin.

To further analyze the expression of serotonergic vesicles, we measured the number of vesicles per cell and the number of serotonergic cells following both treatments. We expected 5-HTP treatments to lead to the production and packaging of more vesicles per cell. We found the increase in vesicle formation following 5-HTP treatment is not due to packaging of more vesicles per cell, as the average number of serotonergic vesicles per cell was not significantly different from control and 5-HTP treated animals (Fig. 7). However, there was a significant ($p < 0.001$) increase in the number of serotonergic cells following 5-HTP treatment (Fig. 8). Elimination of vesicles by treatment with PCPA obviously caused a significant decrease in both vesicles per cell and in serotonergic cells ($p < 0.001$; Fig. 7 & 8). The increase in vesicles following treatment with 5-HTP was due to an increase in the number of cells containing vesicles.

Mechanosensory axon quantification

To quantify how the gain and loss of function experiments affected mechanosensory axon innervation, we digitized and analyzed HNK-1 labeled *camera lucida* tracings. As with serotonergic vesicles, we quantified neurites in dorsal and ventral regions of the skin. First,

we tested whether serotonin levels affected the length of mechanosensory axons innervating the larval epidermis. We hypothesized that 5-HTP treated animals would have longer axon lengths than controls and PCPA treated animals would have shorter axon lengths than controls. Quantification of mechanosensory axon length indicated no change between controls and 5-HTP treated animals, but did show a slight decrease in length following PCPA treatments that was statistically significant in ventral regions (Fig. 9). The ventral region is the farthest from the sensory cell body and would be the region most affected by persistent disruption of targeting signals.

Next, to test the relation between serotonin vesicle production and innervating RB axons we analyzed the number of encircled cells following each treatment. We hypothesized that 5-HTP treated animals would have more encircled cells, while PCPA treated animals would have fewer compared to controls. While not statistically significant in dorsal areas, the number of encircled cells does significantly ($p < 0.05$) increase in ventral areas following 5-HTP treatment (Fig. 10). Encircling in PCPA treated animals is significantly less than in 5-HTP treated animals ($p < 0.001$) in both dorsal and ventral regions (Fig. 10). Encircled cells should be the SSCs seen in double labeling experiments (Fig. 5). Thus, encircled cells are correlated with the amount of serotonin present in SSCs in the skin.

Finally, if serotonin is used to guide mechanosensory axons, its absence could trigger axon retraction. We tested this hypothesis by counting the number of retraction bulbs following each treatment. We expected a larger number of retraction bulbs in PCPA treated animals than in controls since they lack all serotonergic vesicles. The number of

retraction bulbs shows little difference between 5-HTP and controls, but a significant ($p < 0.01$) increase in PCPA treated animals compared to both controls and 5-HTP treated animals (Fig. 11). This suggests serotonin is necessary for the maintenance of normal nerve endings in the developing skin of *X. laevis*.

The developmental function of mechanosensory axons and their targets

Tissue remodeling

The skin changes as the animal goes through its transition from larval to metamorphic stages. At the same time, RB mechanosensory neurons undergo apoptosis and their function is taken over by DRGs. To test whether SSCs also undergo programmed cell death, we utilized the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to visualize epidermal apoptosis. We predicted that SSCs would undergo apoptosis shortly before programmed cell death begins in RB mechanosensory cells. We found small uniform clusters of cells undergoing apoptosis at st. 46 or 4.42dpf (Fig. 12). Therefore, programmed cell death occurs in the skin at the same time as RB death. The dying cells are similar in size to SSCs and form a similar pattern in the skin.

Behavioral changes

To test whether serotonin affected the sensory response to stimuli, we quantified behavior following pharmacological manipulation of serotonin levels in larval tadpoles. RB mechanosensory neurons mediate the escape response in *Xenopus* larvae (Boothby and

Roberts, 1992). We assayed the escape response in normal and treated animals. We hypothesized that embryos incubated in 5-HTP would either show an increase or no difference in escape response compared to controls, but that embryos incubated in PCPA will have a higher proportion of unresponsive or inappropriate escape behaviors. We analyzed whether animals responded and how they responded to a noxious stimulus. The intensity of response to a noxious stimulus was altered by pharmacological treatment. Controls and 5-HTP treated animals displayed no response to the stimulus 5% and 6.1% of the time respectively, while PCPA treated animals displayed no response 22.5% of the time (Fig. 13A). Also, PCPA treated animals showed a lower frequency of the high intensity, swimming response (37.5%) compared to controls (72.5%; Fig. 13A). Furthermore, PCPA treated animals displayed more low intensity flexions (40%) compared to controls (22.5%) (Fig. 13A). Control and 5-HTP treated animals escaped to the contralateral side, or away from the stimulus, 84.2% and 87.1% of the time respectively. While very few tried escaping towards the stimulus. In PCPA treated animals, however, only 48.4% of tadpoles escaped to the contralateral side and a majority moved inappropriately towards the stimulus (Fig. 13B). PCPA treated animals had a much larger percentage of unresponsive trials and more than half of all responses were aberrant with animals moving toward the stimulus. This suggests that the wiring of the escape response circuit was changed.

Figure 2

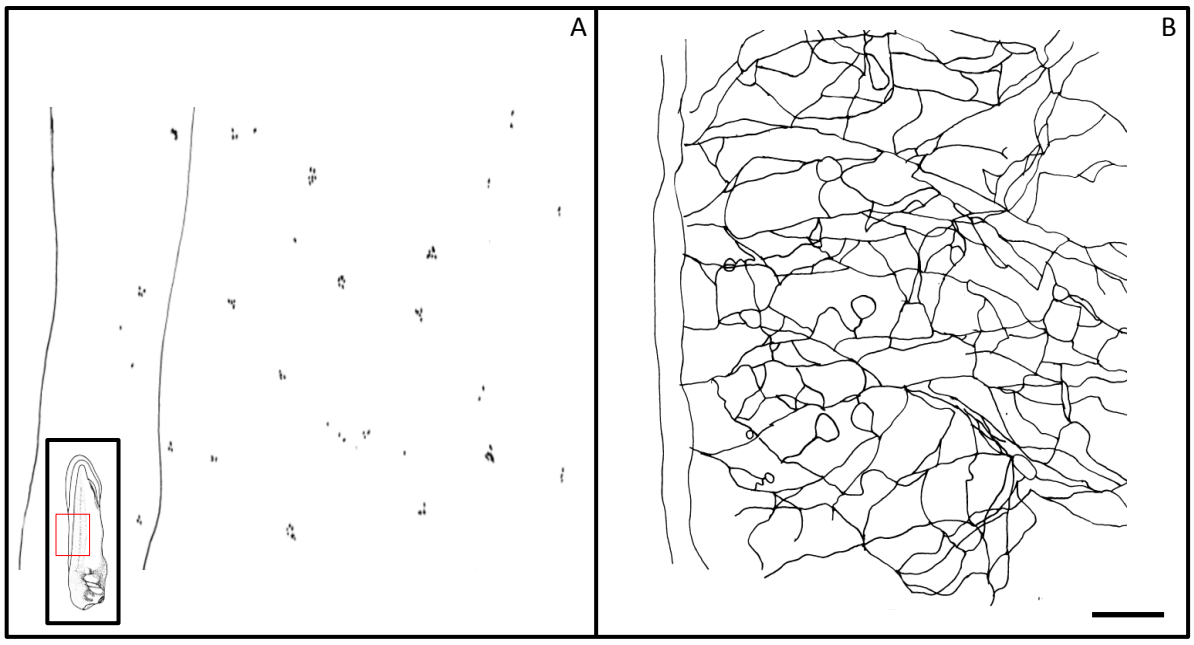

Figure 2: Wild-Type distribution of serotonergic vesicles and mechanosensory axons. (A) *Camera Lucida* tracing of serotonergic vesicle distribution showing clusters in the skin of a control tadpole. (B) *Camera Lucida* tracing of HNK-1 labeled mechanosensory axon distribution with several encircled cells in the skin of a control tadpole. The serotonin-containing vesicles and the RB axons are spread throughout the skin at this stage (33/34, 1.85dpf) of development. Inset – stage, orientation and sampling region of this and subsequent figures. Scale bar: 50µm.

Figure 3

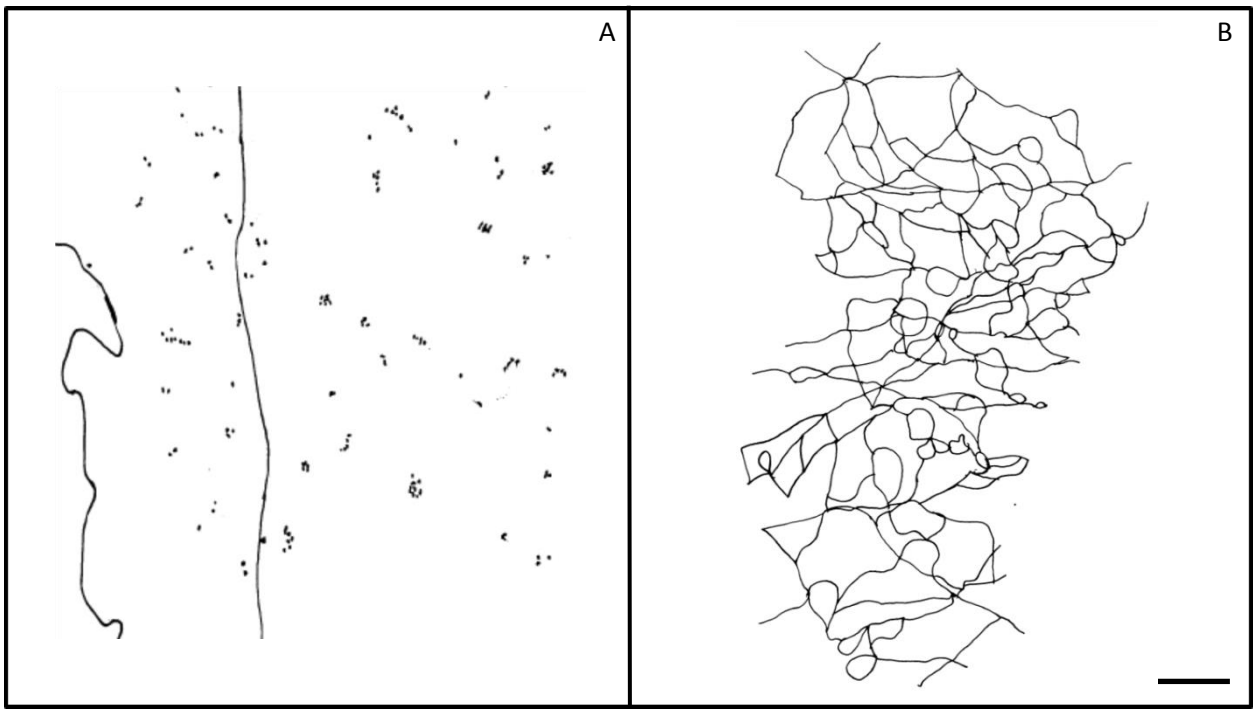

Figure 3: 5-HTP (gain-of-function) treatment leads to increased production of serotonergic vesicles and encircled RB axons. (A) *Camera Lucida* tracing of serotonergic vesicle distribution shows an increased number of vesicles in clusters in 5-HTP treated tadpole. (B) *Camera Lucida* tracing of HNK-1 labeled mechanosensory axon distribution in a 5-HTP treated tadpole shows more encircled cells. Stage, orientation and region same as in Fig. 2. Scale bar: 50µm.

Figure 4

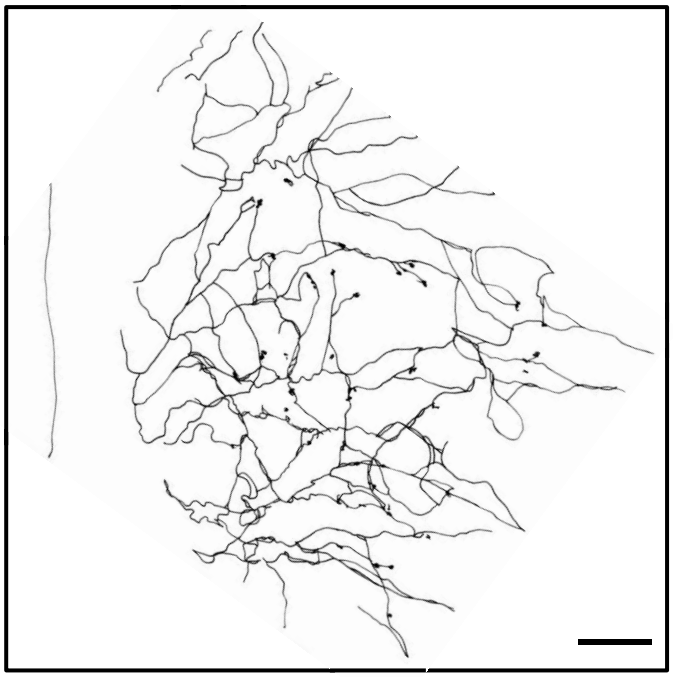

Figure 4: PCPA (loss-of-function) inhibits serotonergic production and leads to aberant mechanosensory axon distribution. *Camera Lucida* tracing of HNK-1 labeled mechanosensory axon distribution in PCPA treated tadpole shows few encircled cells and many axons ending in large retraction bulbs. Stage, orientation and region same as in Fig. 2. Scale bar: 50μm.

Figure 5

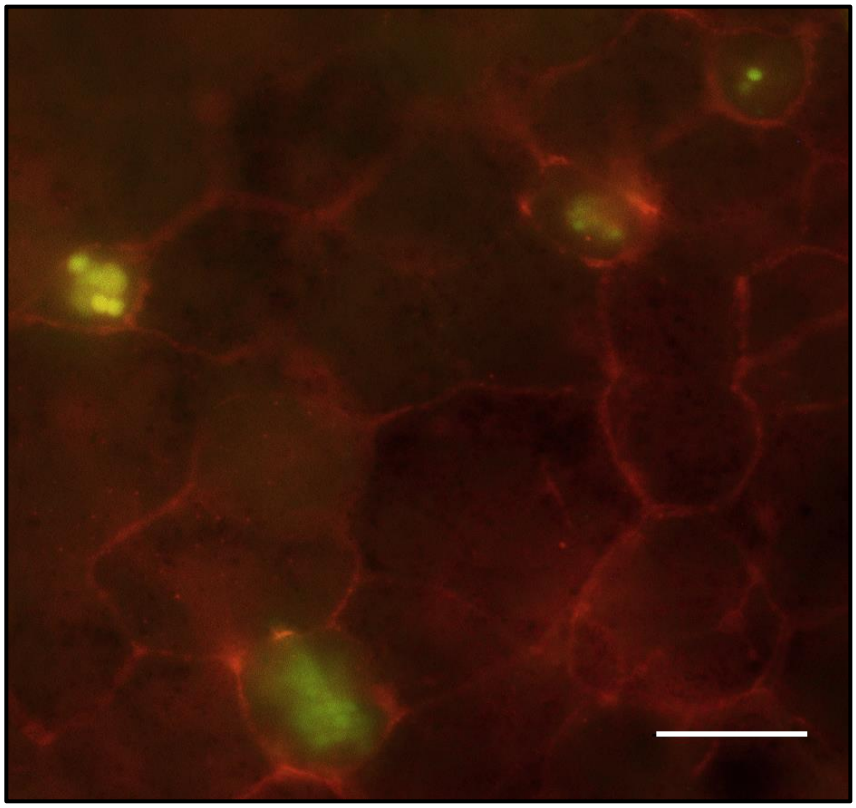

Figure 5: Serotonergic vesicles are encircled by HNK-1 labeled axons in larval skin. Double immunofluorescent labeling: HNK-1 mechanosensory neurons are in red, while serotonergic vesicles are labeled in green. Note cells containing green are encircled by red axons. Scale bar: 50μm. Stage 33/34, 1.85dpf.

Figure 6

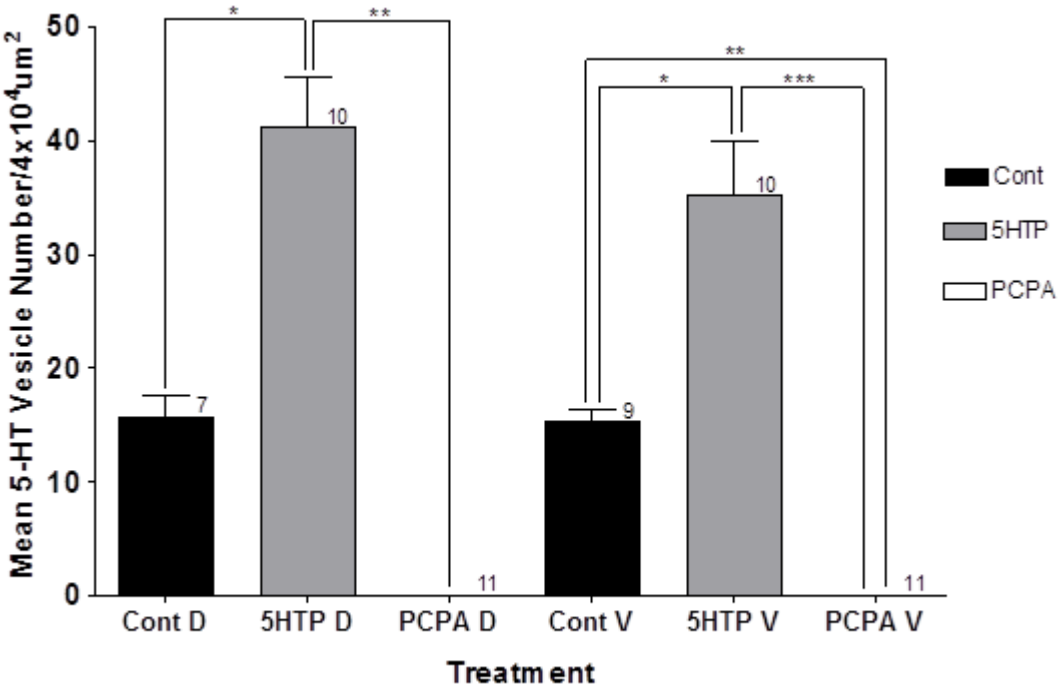

Figure 6: Number of vesicles produced is influenced by the amount of serotonin. Y-axis: average serotonergic vesicles per 40,000μm^2 region. X-axis: the results of treatment in dorsal (D) and ventral (V) areas. In both regions, increasing the amount of serotonin by treating animals with 5-HTP, increases the number of vesicles, while decreasing the amount of serotonin by treating with PCPA, eliminates all vesicles. Numbers represent *n* values for each treatment. *** denotes *p*-value < 0.001, ** denotes *p*-value < 0.01, * denotes *p*-value < 0.05. Error bars represent SEM.

Figure 7

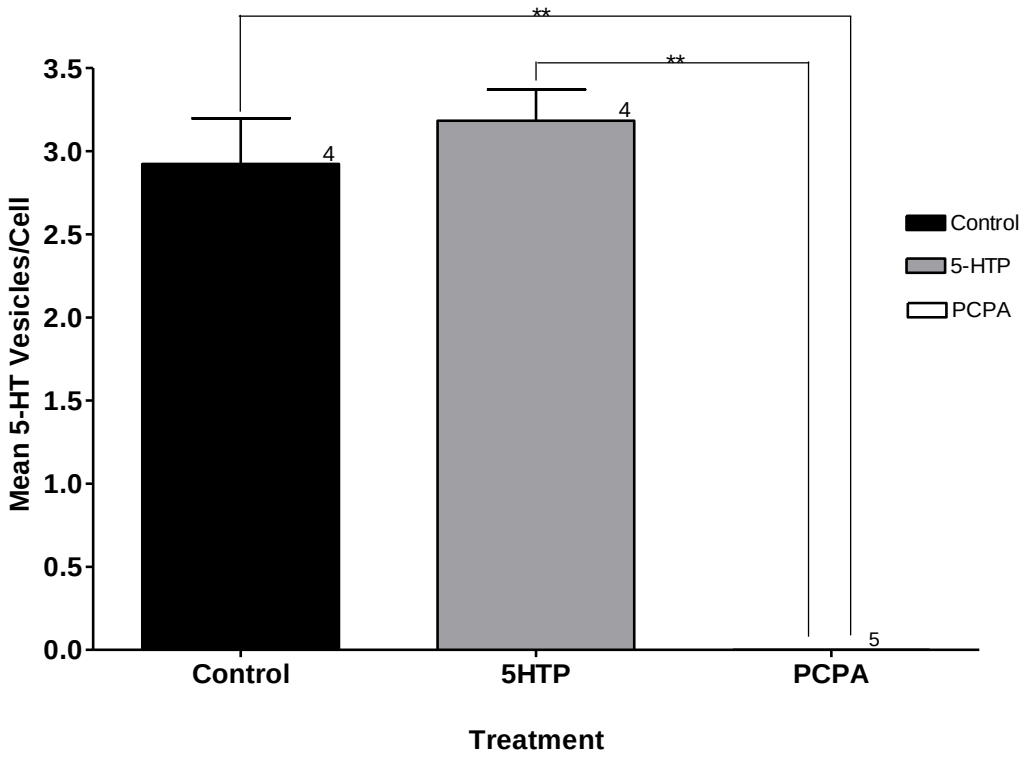

Figure 7: The number of vesicles per cell does not increase following treatment with 5-HTP. Y-axis: average number of serotonergic vesicles per cell in three 40,000μm^2 regions. X-axis: the results of treatment in all dorsal regions. The 5-HTP gain-of-function had no effect on the number of vesicles per cell, but the PCPA loss-of-function, totally eliminated the vesicles in the skin. Numbers represent *n* values for each treatment. ** denotes *p*-value < 0.001. Error bars represent SEM.

Figure 8

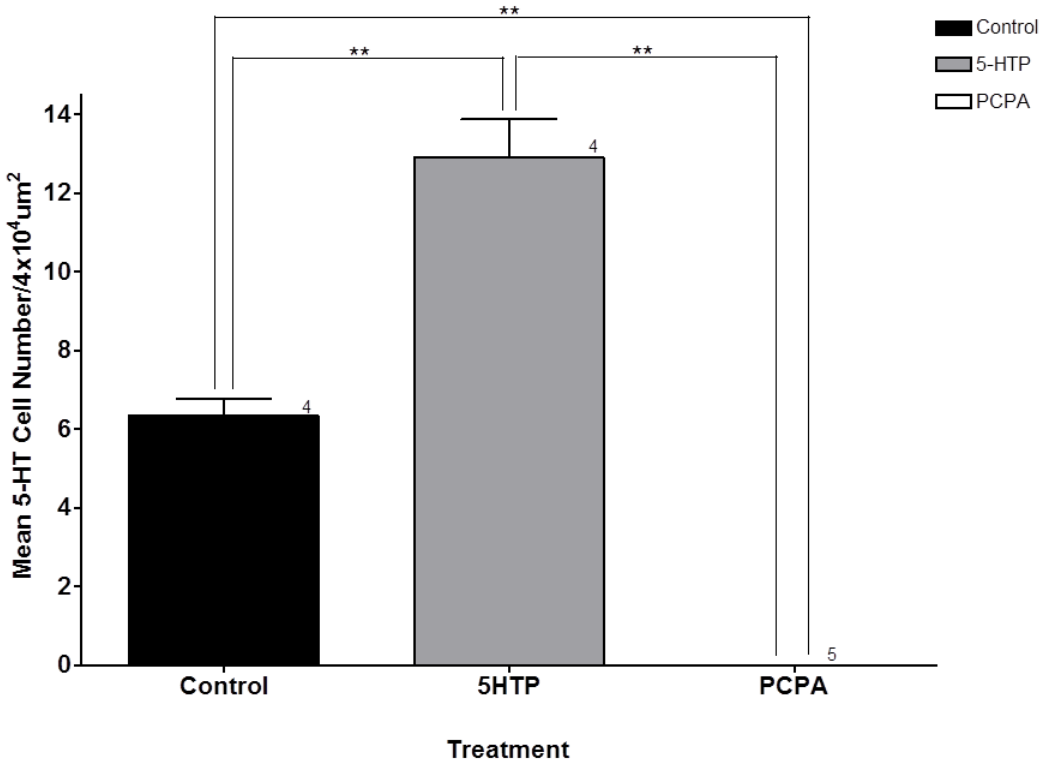

Figure 8: The number of serotonergic cells increased following treatment with 5-HTP. The increase in number of vesicles seen in 5-HTP gain-of-function experiments is due to an increased number of cells containing vesicles. The loss-of-function treatment (PCPA) eliminated cells with vesicles. Y-axis: average serotonergic cells in three 40,000μm^2 regions. X-axis: the results of treatment in all dorsal regions. Numbers represent *n* values for each treatment. ** denotes *p*-value < 0.001. Error bars represent SEM.

Figure 9

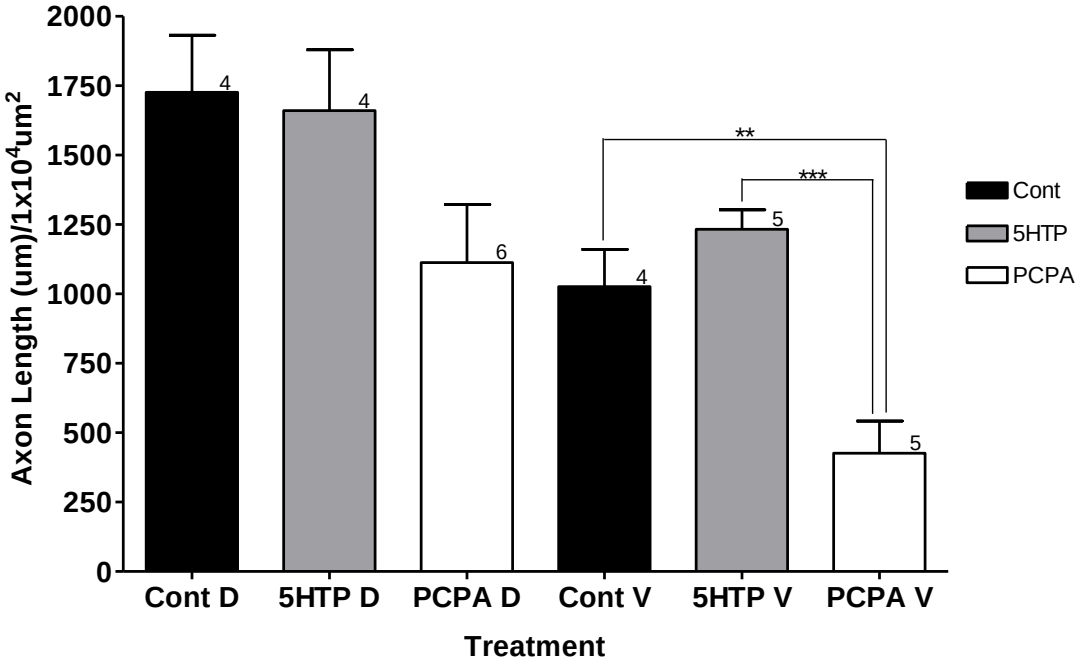

Figure 9: Regional differences in total axon length. The PCPA loss-of-function only decreases total mechanosensory neurite length in ventral regions. This could be related to the greater distance of growth cones from ventral regions. They could be more dependent on guidance cues at this stage of development. Y-axis: average total axon length per 10,000 μm^2 region. X-axis: treatment and area, dorsal (D) or ventral (V). Numbers represent *n* values for each treatment. *** denotes *p*-value < 0.001, ** denotes *p*-value < 0.01. Error bars represent SEM.

Figure 10

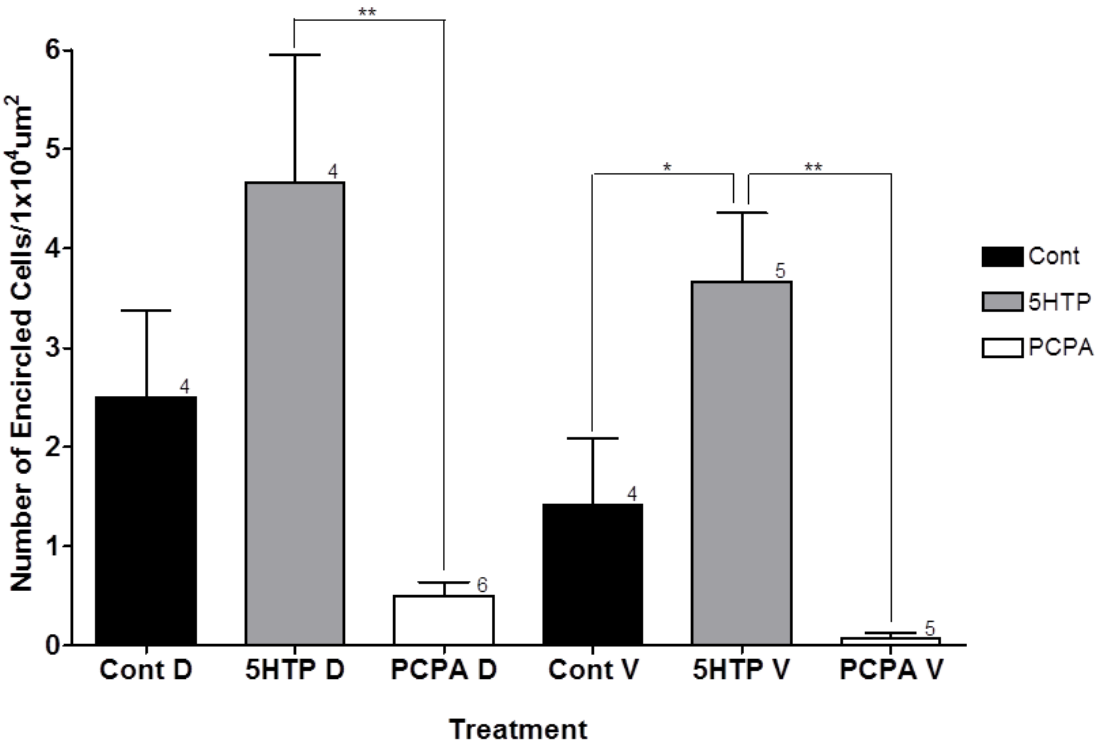

Figure 10: Pattern of innervation. Encircling by RB mechanosensory axons increases with 5-HTP treatment and decreases with PCPA treatment. The encircled cells are the same size and distributed in the same pattern as SSCs. Y-axis: number of encircled cells per 10,000 μm^2 region. X-axis: treatment and area, dorsal (D) or ventral (V). Numbers represent *n* values for each treatment. ** denotes *p*-value < 0.01, * denotes *p*-value < 0.05. Error bars represent SEM.

Figure 11

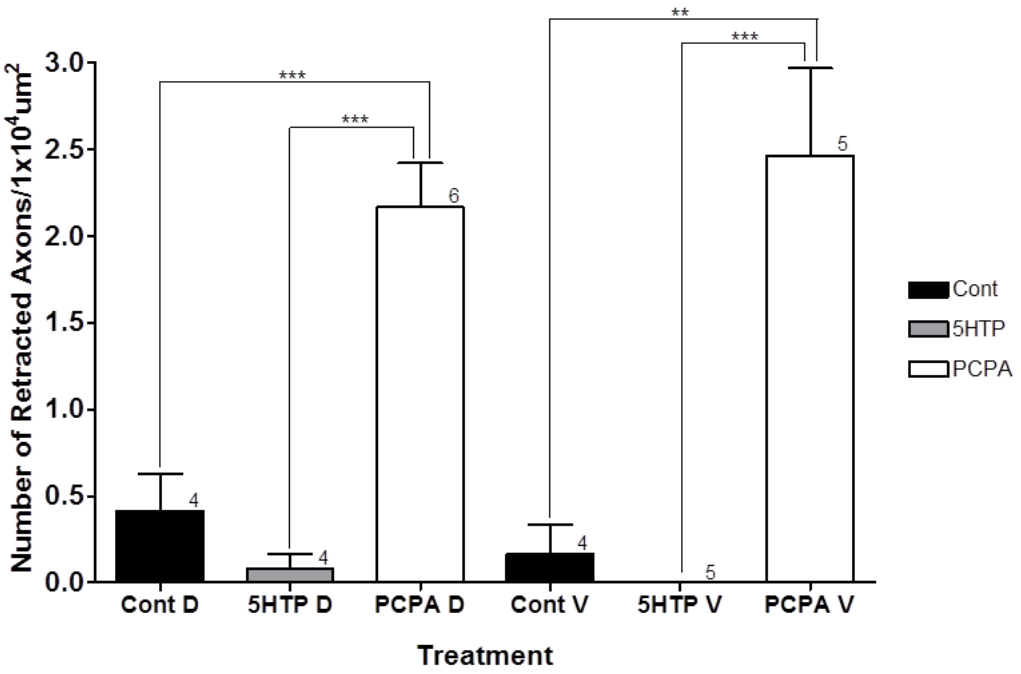

Figure 11: Serotonin and sensory axon retraction. PCPA treated loss-of-function animals had increased evidence of mechanosensory axon retraction. The number of retraction bulbs increased in the absence of serotonin. Y-axis: number of retracted axons per 10,000 μm^2 region. X-axis: treatment and area, dorsal (D) or ventral (V). Numbers represent *n* values for each treatment. *** denotes *p*-value < 0.001, ** denotes *p*-value < 0.01. Error bars represent SEM.

Figure 12

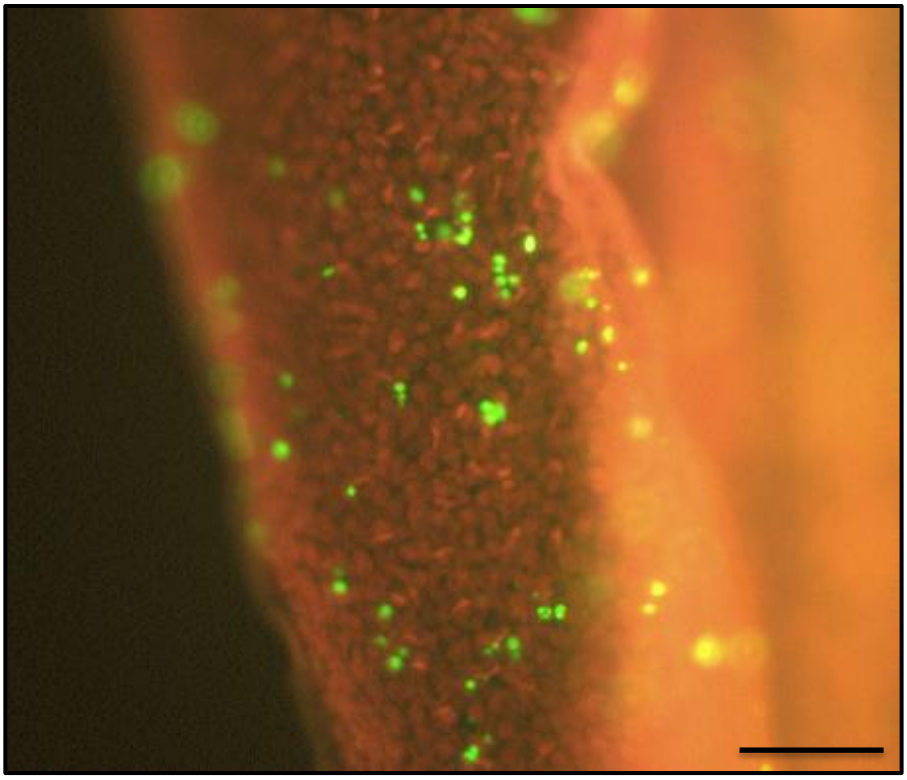

Figure 12: Programmed cell death in the epidermis. Just before metamorphosis, epidermal cells undergo programmed cell death. The cells are the size of SSCs and occur in a scattered pattern reminiscent of SSCs. TUNEL labels dying cells in green while propidium iodide marks living cells in red. Scale bar: 50μm. Stage 46, 4.42dpf.

Figure 13

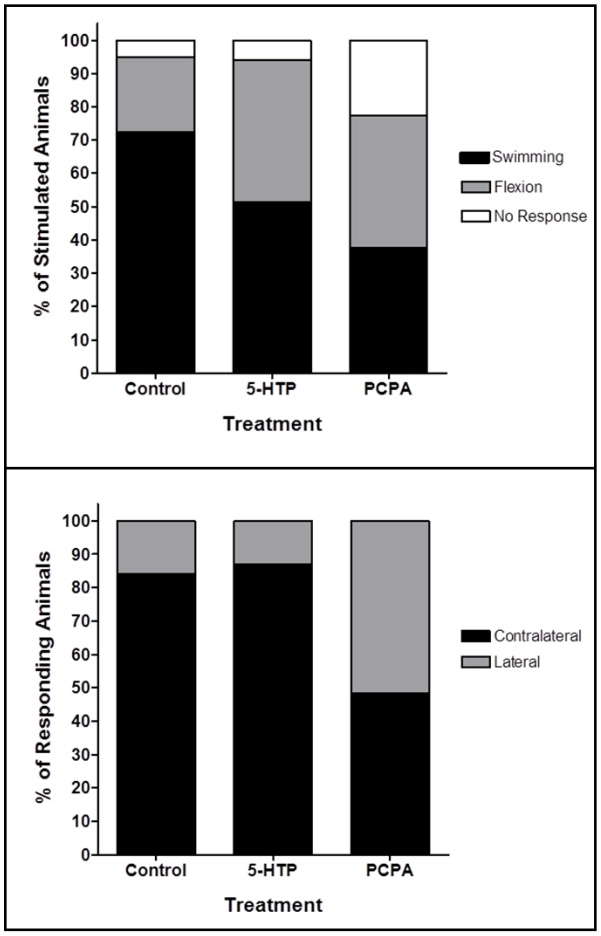

Figure 13: Serotonin influences the escape response in larval *X. laevis*. A. Animals responding to a noxious stimulus. When serotonin is reduced by PCPA, fewer animals respond and the majority of those responding exhibit a less responsive flexion rather than the more active swimming away from the stimulation. B. Direction of response. When serotonin is reduced by PCPA, the majority of animals move toward rather than away from the stimulus. The inappropriate response indicates the escape circuit is not properly connected. Y-axes: percentage of animals displaying each response. X-axes: Treatment. Bars show stacked responses of the possible outcomes. Control n = 40, 5-HTP n = 33, PCPA n = 40

Discussion

Mechanosensory Neuron Development

The emergence of a mechanosensory network in larval amphibians is crucial to their proper development and survival. We've examined the emergence and pattern of innervation of RB neurons, the primary mechanosensory input for *Xenopus laevis* embryos. Our hypothesis is that a recently discovered cell type in *X. laevis* skin plays a chemoattractive role in the innervation of these primary mechanosensory neurons. RB cells arise from the spinal cord and send their growth cones ventrally to innervate targets in the growing epidermis (Roberts and Clarke, 1982). Most RB cells originate during gastrulation (Lamborghini, 1980). These neurons provide mechanosensory input to the frog throughout embryonic and larval stages (Roberts and Smyth, 1974) before starting to undergo apoptosis around the beginning of metamorphosis (Lamborghini, 1987). During metamorphosis, DRG neurons take over the mechanosensory responsibilities in *X. laevis* and also innervate nociceptors, proprioceptors, and thermoreceptors (Gandhi, 2014).

As they are the first sensory neurons to differentiate, it is crucial that RB processes be evenly distributed throughout the embryonic skin. According to Roberts and Taylor (1989) RB growth cones move up to 80μm per hour at the peak of innervation and will provide the larvae with critical information about their immediate surroundings, including threats from predators, at this stage of development. The pattern of innervation by RB neurites has been studied by many different investigators over the years (Roberts and Taylor, 1982; Jacobson and Huang, 1985; Nordlander, 1989). During embryonic development, RB neurons form in two longitudinal columns of the neural tube (Rohon,

1888; Beard, 1889; Hughes, 1957; Chitnis et al. 1995) and sensory axons are guided to the periphery to form a plexus providing sensory input to the embryo (Roberts and Taylor, 1982; Nordlander, 1989; Wieczorek, 2002). Once established, this plexus is comprised of evenly distributed axons intertwining and encircling some larval skin cells. This encircling pattern of innervation is of particular interest, as it is distributed throughout the embryonic and larval skin.

In order to ensure proper innervation, growth cones need to be guided to their targets. Possible cues include physical guidance, or stereotropism, which involves growth cones being guided by channels or tunnels to direct innervating sensory axons (Weiss, 1934; Ebendal, 1976; Silver and Robb, 1979). Stereotropism, while important, is not the only factor in axon guidance. Most growth cones receive molecular signals, or guidance cues, from their targets. These can be in the form of extracellular matrix molecules, cell surface molecules, or diffusible tropic factors, which attract or repel growing axons (Dodd and Jessell, 1988).

Development of Small Secretory Cells

Recent work has shed light on the targets of RB neurons and their distinct encircling pattern of a specific subset of epidermal cells (Somasekhar and Nordlander, 1997; Walentek et al. 2014). Conical cells are a novel cell type identified within the epidermis of *X. laevis*. These cells can synthesize and secrete serotonin and have been renamed Small Secretory Cells (SSCs; Walentek et al. 2014). There has been speculation on the function of these cells. First, Somasekhar and Nordlander, (1997) hypothesized that RB neurites are

attracted by the lack of chondroitin 6-sulfate (C6S) since it repels growth cones. The lack of C6S in the SSC basal lamina may make it easier for axons to stick to the cell surface of SSCs rather than other skin cells which contain C6S at this time. Dubaissi et al. (2014) showed that SSCs protected animals from bacterial infection, and Walentek et al. (2014) showed that these cells were also required for ciliary beating in another class of skin cells needed for mucus transport. SSCs may very well have other functions in the larval epidermis of *X. laevis*, and we propose a role for them in patterning mechanosensory RB axon innervation. We hypothesize that RB axons encircle SSCs and that the production and secretion of serotonin plays a role in guiding the initial outgrowth and spacing of sensory neurites.

SSC differentiation has not been investigated in detail, but much is known about the patterning and development of other types of *X. laevis* skin cells. One class, multi-ciliated cells (MCCs), undergo a two-step process to develop their pattern and spacing in the skin. First, they differentiate into ciliated-cell precursors in the inner layer of the non-neural ectoderm. They then intercalate into the superficial layer of the epidermis from the deep layer of the ectoderm during early gastrulation and are evenly spaced in the larval skin (Deblandre et al. 1999). Since SSCs also arise from the deep ectoderm layer and intercalate into the superficial layer, they might use a similar patterning mechanism. Our work suggests they are similarly patterned and spaced within the superficial epidermal layer.

The first evidence of SSC differentiation starts with the production of serotonin in the skin. It can be detected with immunolabeling in *X. laevis* epidermal tissue around st. 26 (1.23dpf), but it is not plentiful at that time (Walentek et al 2014). At st. 33/34 (1.85dpf), serotonin levels have increased and labeling shows a punctate pattern within the skin.

Walentek et al. (2014) showed that mRNA expression of slc18a1, a vesicular monoamine transporter occurs in *X. laevis* larval skin, suggesting that the dense granules are vesicles containing serotonin. Our data show that once packaged in SSCs, serotonin appears to be chemoattractive to RB mechanosensory axons since it promotes growth in ventral skin regions and causes more skin cell encircling. After this, serotonin containing vesicles are slowly pushed to the apical surface of SSCs where they are secreted onto the skin. Once there they affect the ciliary beating of neighboring skin cells involved in mucus transport (Walentek et al. 2014).

SSCs or conical cells, appear to be the first larval skin cells innervated by primary RB mechanosensory axons (Somasekhar and Nordlander, 1997). Other classes of skin cell types have RB neurites contacting them, but are not fully encircled like SSCs. This conclusion is supported by our double immunofluorescent labeling of RB axons and serotonin containing SSCs. Futhermore, Somasekhar and Nordlander (1997) found granular peroxidase reaction products inside their conical cells. These granular products would be the serotonin containing vesicles seen in our double labeling studies.

Pharmacological Manipulation

In order to investigate a role for serotonin in the innervation of primary mechanosensory RB axons, we pharmacologically manipulated its levels in *X. laevis* larval skin. We were able to alter serotonin levels in the skin and quantify the sensory axon response. In ventral areas of the animal, overall axon length decreased in treatments that resulted in elimination of serotonin, supporting a role for it in the development of

mechanosensory innervation. Furthermore, treatments eliminating serotonin also lower the number of SSCs encircled by neurites originating from RB neurons. While many skin cells are still innervated, serotonin is needed for proper innervation of SSCs by the mechanosensory RB processes. Another key difference is that when serotonin is reduced, there is an increase in the number of retraction bulbs found in larval *X. laevis* skin, suggesting that serotonin is necessary for normal maintenance of RB neurites innervating the developing epidermis.

Serotonin has a well-known role in developmental processes. It takes part in the migration of axons in *C. elegans* (Kindt et al. 2002) as well as guidance of specific axon types in mammals (Speranza et al. 2013). Furthermore, modifying serotonin levels in embryonic mouse brain leads to the disruption of sensory maps of thalamocortical axons (Bonnin et al. 2007). Daubert and Condron (2010) also showed that there is a critical developmental window when altered serotonin levels can permanently impact sensory circuitry as well as contribute to a number of neurodevelopmental and neuropsychiatric disorders. All of these are consistent with a chemotropic role for serotonin in axon guidance.

There are also other skin cells associated with mechanosensory axon innervation. Merkel cells, a skin cell type associated with mechanosensory axons, attract Dorsal Root Ganglion (DRG) processes during regeneration in amphibians (Scott et al. 1981). Though not necessary for mechanosensory transduction, Merkel cells have been found in all vertebrates and provide targets for growing mechanosensory nerves, ensuring the proper distribution of sensory neurites in amphibians, including *Xenopus* (Mearow and Diamond,

1988). Even in invertebrates, sensory neurons are attracted and organized in a complex network during embryonic development (Baratte and Bonnaud, 2009). They are then sent to innervate their final targets and establish a clear pattern for the early peripheral nervous system. Furthermore, leukocyte common antigen-related (LAR) receptors on projecting RB neurons in *D. rerio* are attracted to membrane bound heparin sulfate proteoglycans (HSPGs) in the skin (Lee et al. 2004). The expression of LAR receptors is conserved in sensory neurons (Schaapveld et al. 1998), so it is plausible that a similar attractant system might be seen in *Xenopus*. We hypothesize that serotonin in the SSCs is a long range signal for mechanosensory RB axons. Since SSCs are found evenly spaced throughout the superficial epithelial layer, their attraction of sensory axons could ensure that the animal would have sensory innervation evenly distributed throughout the larval skin (Gandhi, 2014).

Functional Consequences

After pharmacological experiments provided evidence of successful serotonin manipulation, we focused our attention on functional consequences related to this modification. If, in fact, we were able to disrupt the normal innervation of RB mechanosensory neurons by reducing the amount of serotonin present in the skin, then we should also see an aberrant sensory response in those animals. We hypothesized that eliminating serotonin in larval epidermis would lead to incorrect escape responses.

Escape responses are extremely important in hatchling animals. They provide the embryo with a way to escape predation at early stages of development. *X. laevis* embryos

usually escape or swim away from an external stimulus applied to their skin. The animal first turns or orients itself away from the stimulus, and then proceeds to swim in this new direction until it is clear of the threat. Another, less frequent, response to an external stimulus applied to the skin is a flexion. *X. laevis* larvae display two different degrees of flexion, a more extreme C-flexion, and a lesser V-flexion, in response to a noxious external stimulation (Roberts et al. 2010).

Preliminary experiments to test escape responses after pharmacological manipulation showed that tadpoles with reduced serotonin in their skin, responded less frequently to the stimulus than controls. They also showed a higher frequency of flexions compared with controls and a lower frequency of swimming. Absence of serotonin appears to have an effect on the magnitude of escape responses observed. Furthermore, these treatments show a difference in the direction of the escape response. A larger percentage of loss of function treatments showed incorrect escape responses in animals still capable of responding to a noxious stimulus. Incorrect responses had animals moving towards an external stimulus rather than away from it. Other investigators have found a similar percentage of escapes to the contralateral side of stimulation in control animals as well as the percentage of animals displaying the predominant swimming response (Boothby and Roberts, 1992). Our data, while preliminary, suggest that incorrect innervation of the skin by RB neurons can result from a lack of serotonin in the larval epidermis and that it leads to an aberrant and potentially lethal set of behaviors.

Tissue Remodeling

Massive changes in the skin occur during the transition from embryonic to larval to metamorphic and ultimately to adult stages in *X. laevis.* Our final investigation was concerned with the larval to metamorphic transition. After only 7 days of induced metamorphosis via thyroid hormone (TH) treatment, 401 genes were differentially expressed by more than fourfold in *Xenopus* skin (Suzuki et al. 2009). To accommodate this large-scale reorganization of tissue, many cells undergo programmed cell death (PCD), or apoptosis within the skin. A multitude of cells also undergo PCD in the trunk and tail region due to tail resorption and the animal experiences a transition in muscles from larval to adult (Nishikawa, 2012).

Remodeling during metamorphosis is not confined to the skin, it also affects sensory neurons. RB neurons begin undergoing apoptosis around the start of metamorphosis (Lamborghini, 1987) and their sensory function is assumed by DRG neurons. This transition is a gradual one that ends with a complete overhaul of sensory nerve endings in the adult frog. DRG axons innervate some of the same targets as RB neurons and are also attracted by similar cues. In a similar manner, DRG axons are attracted to Merkel cells during regeneration (Scott et al. 1981). This relationship between adult mechanosensory neuron and adult sensory skin cell is similar in nature to the one described here, between embryonic RB mechanosensory neurons and SSCs.

We hypothesize that another change occurring during this metamorphic transition is the elimination of SSCs. Previous literature describes the packing, synthesis, and secretion of serotonin from SSCs, but does not address the fate of the cells themselves. If RB

mechanosensory neurons undergo apoptosis, then perhaps SSCs also undergo PCD around this same point in development. Utilizing the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to visualize epidermal apoptosis, we found small uniform clusters of cells undergoing PCD around the same time RB neurons begin apoptosis (Fig. 12). The dying cells are the same size and shape as SSCs. They are also dispersed in a similar pattern throughout the epidermis.

We propose a model for the role of SSCs in patterning the innervation of the developing skin. First, secretion of serotonin by SSCs attracts the long-range movement of RB growth cones to the skin during embryonic development (Fig. 14A). Second, continued secretion leads to "homing" and encircling of the SSC by RB processes and maintenance of the neurites during larval life (Fig. 14B). Finally, SSCs undergo apoptosis, leading to retraction of the RB neurites and perhaps their apoptosis as the animal prepares for metamorphosis (Fig. 14C). During the period that RB neurons innervate the SSCs, mechanosensory neurons from the DRGs grow to an emerging population of specialized Merkel cells in the skin and receive mechanosensory innervation throughout the rest of the animal's life. Of course, the growth of the animal will require the addition of more mechanosensory neurons, making sensory innervation a dynamic process that continues throughout the life of the animal. Thus, the growth of neurons to new targets and the target providing for the maintenance of the neurons, could provide a theme for mechanosensory innervation mediated by different systems at different stages of development.

Figure 14

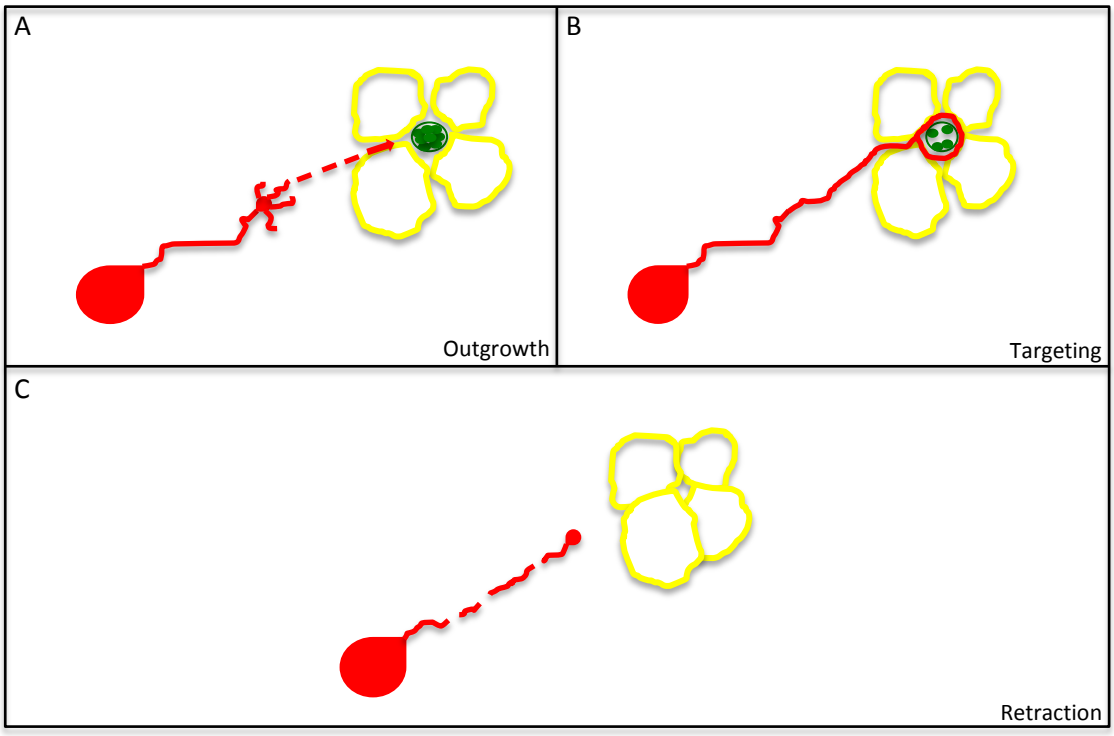

Figure 14: Proposed model of development of mechanosensory innervation in *X. laevis*. (A) RB cell body with axon and growth cone (red) moving over a long range to an SSC with serotonergic vesicles inside (green) surrounded by epidermal cells (yellow). (B) RB axon using short-range targeting to completely surround an SSC during larval skin innervation. (C) SSC undergoes apoptosis and a retraction bulb forms on the RB axon that initiates programmed cell death within the RB cell.

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