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Development of Peripheral Innervation in the Frog *Xenopus Laevis*

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DEVELOPMENT OF PERIPHERAL INNERVATION
IN THE FROG *XENOPUS LAEVIS*

by

Mitali A. Gandhi

A Thesis Submitted in
Partial Fulfillment of the
Requirements for the Degree of

Master of Science
in Biological Sciences

at

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August 2014

ABSTRACT

DEVELOPMENT OF PERIPHERAL INNERVATION IN THE FROG *XENOPUS LAEVIS*

by

Mitali A. Gandhi

The University of Wisconsin-Milwaukee, 2014
Under the Supervision of Dr. R. David Heathcote

The skin in *Xenopus laevis* is innervated by two different sets of mechanosensory neurons at different times during development. Rohon Beard (RB) neurons start differentiating during gastrulation, innervate the embryonic skin and mediate sensory function during hatching. Dorsal Root Ganglion (DRG) neurons start differentiating after neural crest migration, innervate adult epidermal targets and mediate mechanosensory function during larval and adult stages and eventually replace RB neurons. The change in sensory neurons occurs during the transformation of skin, sensory structures, and behavior from their embryonic to their larval forms. We hypothesized that developmental changes in either the sensory end organs or the skin underlie the switch in mechanosensory systems. We initially tested the development of sensory innervation of the skin by marking neurites that innervate the skin and measuring their spatial and temporal changes. We also showed that sensory neurites rapidly disappear following denervation. We then switched targets by transplanting denervated skin between animals of the same stage (isochronic) and animals at different stages (heterochronic). Quantification of the percent area reinnervated by neurites from the host showed that reinnervation of all three stages of larval development following isochronic transplant,

was approximately half that of controls. Reinnervation of heterochronic transplants was approximately half of the isochronic transplants. Our results show that neurites have difficulty innervating denervated skin at an age different from itself. In addition, the pattern of innervation changes during this period of development. Sensory neurons totally encircle a subset of dispersed cells in the skin. Innervation of other cells follows, but the number of encircled cells gradually decreases. Following heterochronic skin transplants, embryonic neurites encircle cells in a similar way in both embryonic and larval skin, indicating that the neurons, rather than the skin determines the pattern of innervation. However, older neurons do not show age-specific patterning, which suggests that they no longer determine the pattern of innervation. Thus, the skin targets help regulate target innervation, but the patterning of innervation, at least partially depends on the neurons.

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Introduction

As an animal develops, its needs change, requiring the activity of different sets of neurons. In most animals, the embryo is encased in an egg from which it must escape to become a freely-moving and independent larva. In fish and amphibians, different populations of sensory neurons form during these periods of development. The first or primary sensory neurons differentiate during embryonic development and mediate the mechanosensory functions needed for hatching. The secondary sensory neurons differentiate after hatching. They gradually assume the mechanosensory functions and provide additional sensory information (proprioception, thermoreception, nociception) that is essential for animals at later stages of their development. We test whether the change in skin targets of the mechanoreceptors drives the formation of and switch to the secondary neurons by transplanting the skin and its associated targets between animals at two different stages of development.

Sensory Neuron Origin: Primary and secondary sensory neurons have distinct spatial patterns of expression. Primary sensory neurons originate from the lateral edges of the developing neural plate and become located in the dorsal neural tube following neural fold fusion. The cells are quite large and are called Rohon Beard (RB) neurons, after two of the early researchers who studied them (Rohon, 1885; Beard, 1889, 1892, 1896). Secondary sensory neurons are derived from the embryonic neural crest and ultimately differentiate in the Dorsal Root Ganglia (DRG) associated with each segment. The region of ectoderm that is between the lateral neural plate and the dorsal epidermis

forms the neural crest. Following fusion of the neural folds, the neural crest is deposited on top of the neural tube. Cells then begin to migrate to their peripheral locations. The first cells to migrate have distal destinations and form the sympathetic ganglia. Subsequent neural crest cells do not migrate as far from the neural tube and differentiate into the DRGs.

There are also distinct temporal patterns of expression of primary and secondary sensory neurons. The final cell divisions or “birthdays” of the population of primary RB neurons, start during gastrulation and continue during neural plate formation (Lamborghini, 1980), while DRG neurons are thought to undergo their birthdays much later, during and after their migration from the neural crest (Lawson et al, 1974).

Although the time and place of differentiation of the two populations of sensory neurons is distinct, their origins are linked. Both are mixed in the border region between the neural plate and ectoderm (Artinger et al, 1999; Cornell and Eisen, 2000, 2002) and the fate of both are induced by BMP signaling (Rossi et al, 2008). Both sensory neuron types are also dependent on the expression of the Neurogenin-1 transcription factor and Delta signaling (Cornell and Eisen 2000). These results have led to the conclusion that RBs and Neural Crest Cells may have a related origin, but they do not necessarily have a common progenitor (Cornell and Eisen, 2002).

Sensory Neuron Differentiation. The differentiation of all sensory neurons follows a similar sequence. The pathways for both RB and DRG neurons start with the expression of the basic Helix-Loop-Helix transcription factor, Neurogenin-1 and/or -2 (Ma et al, 1996, Fode et al, 1998) followed by Neurod-1 and -4 (Fode et al, 1998). After

this neurogenic phase, the cells stop dividing and begin expressing the transcription factors Islet-1 and Brn3a (Anderson, 1999). The expression of Islet-1 in all sensory cells is a necessary step in the specification of all types of sensory neurons (Sun et al, 2008).

Following specification, the neurite outgrowth phase of differentiation begins. In *Xenopus*, RB neurons are among the first to initiate neurite outgrowth, and do so around the time of neural tube closure (Hayes and Roberts, 1973; Hartenstein, 1989; Nordlander, 1989). They send neurites along the neural tube and into the periphery where they form a dense plexus underlying the embryonic epidermis (Roberts and Hayes, 1977; Roberts and Taylor, 1982; Taylor and Roberts, 1983; Somasekhar and Nordlander, 1997; Wieczorek, 2002). During peripheral innervation, they surround some cells in the skin (Somasekhar and Nordlander, 1997). Eventually nerve endings are present on all epidermal cells, but they appear to totally surround only a subset of epidermal cells arranged in a dispersed pattern.

Although they differentiate at a later stage of development, DRG neurons also extend neurites during the early phase of their differentiation. After hatching, DRG neurons begin to differentiate and send neurites to the central nervous system and the periphery (Oblinger and Lasek 1994). Although the pattern of innervation of the skin does not obviously change when the DRG neurites first enter, it does undergo a gradual and ultimately dramatic transformation by the time animals start metamorphosis (unpublished observations).

Changes in the sensory innervation occur during the transformation of embryonic to larval skin. This is also the time that larvae start to undergo active swimming and

feeding. Sensory neurons, their targets in the skin, and behavior, all change dramatically over this short period of developmental time.

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).

HNK-1 Marking of Differentiating Sensory Neurons

Animals were anesthetized in 1 mM Tricaine and then fixed before visualizing the sensory neurons. 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 % H₂O₂ 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 monoclonal HNK-1 antibody (Miles Epstein, UW Madison, C0678 from Sigma Aldrich) diluted 1:500 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 1:500 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(Cl₂)₂ and Ni(NH₄)₂(SO₄)₂ was catalyzed by H₂O₂ and used to visualize the HRP

complex. The embryos were washed, infiltrated in 90% glycerol in PBS, whole-mounted in Sylgard wells on microscope slides (Sive et al., 2000) and viewed with a Zeiss Axioskop using brightfield and DIC optics.

Islet-1 Marking of Sensory Nuclei

Immunocytochemistry for Islet-1, a transcription factor expressed in sensory neurons (Korzsh et al., 1993; Sun et al., 2008) was used to mark the nuclei of Rohon Beard (RB) and Dorsal Root Ganglion (DRG) neurons. We used the same protocol as with HNK-1 immunocytochemistry except the primary antibody was a mouse monoclonal to Islet-1 (39-405; Developmental Studies Hybridoma Bank) diluted 1:500.

Skin Denervation

A patch of skin from the trunk region was denervated by using electrolytically sharpened tungsten needles to remove it from the animal. It was maintained in 0.5X Modified Barth's solution (MBS) and 50 µg/ml gentamycin before fixation. Controls were fixed before skin removal. Immunocytochemistry for HNK-1 was then used to mark the neurites in the isolated patches of skin.

Skin Transplants

Patches of skin were transplanted from donors to hosts of the same stage that had a patch of skin removed (isochronic transplants). They were also transplanted between animals at different stages (heterochronic transplants). The animals were maintained in a solution of 0.5X MBS, 50µg/ml gentamycin and 1mM Tricaine, pH 7.45 during the

transplant procedure. The transplanted skin was held in place with a small piece of glass cover slip for an hour, to promote adherence to the new site. The animals recovered for 24 hours in a solution of 0.5X MBS and 50 µg/ml gentamycin before fixation and immunocytochemistry for HNK-1. The stages used for experiments and rationale are summarized in Table 1.

Analysis

Neurite measurements

To quantify the peripheral innervation of the skin during development, we used camera lucida to trace neurites from somite 7 (future fore-limb), somite 10 (inter-limb region), somite 13 (future hind-limb) and somite 22 (tail region). The total length of neurites (peripheral innervation) in each of the somites was measured with a digitizing tablet (Drawing Board VI, GTCO Calcomp) and Sigma Scan Pro software. In the same way, we quantified neurites from experimental and surrounding (control) regions.

To quantify the area innervated by neurites in a patch of skin, we used camera lucida tracings of the patch. The innervated region was enclosed by a polygon and its area was measured with a digitizing tablet. This was compared to the area of the entire patch to obtain the percent area innervated.

Cell counts

The RB cells in the dorsal spinal cord and the DRG cells in the peripheral nervous system that were associated with the corresponding somites (7, 10, 13, and 22) were quantified from camera lucida tracings.

The skin cells encircled by neurites within a given area were identified from camera lucida tracings and counted.

Graphing and statistical analysis (unpaired t-test) were performed using Prism Software (Graphpad).

RESULTS

Pattern of innervation during development

During larval development of the frog, *Xenopus laevis*, there are two populations of neurons that innervate the skin. The Rohon Beard (RB) neurons form in the central nervous system during embryonic development while Dorsal Root Ganglion (DRG) neurons form during larval development and eventually replace the RB neurons.

To test whether the peripheral pattern of innervation changes as the primary sensory neurons (RB) are replaced by the secondary ones (DRG), we marked their neurites with immunocytochemistry to the HNK-1 epitope and visualized the innervation of the skin at different times and locations in the animal. Neurites innervate the skin by growing around the base of skin cells and forming small varicosities or boutons along their length (Fig. 1A). Some cells are entirely surrounded by neurites. The skin innervation was examined at 2.2 d (st 37/38; post-hatching), 3.1 d (st 41; mid-larval stage) and 7.5 d (st 48; start of metamorphosis). It was also examined at four different axial levels, corresponding to the regions where the limbs will form (somites 7 and 13), the interlimb region (somite 10) and the region in the middle of the tail (somite 22). The largest differences were in the tail region (Fig. 1 B). The tail somites undergo a large increase in size during larval development, however the innervation surrounding individual skin cells appears remarkably similar.

Spatial and temporal expression of sensory neurons

We quantified the spatial and temporal expression of both populations of sensory neurons and their peripheral processes to test the coordination of their growth during

larval development. Initially, we used immunocytochemistry for the pan-sensory neuron marker, Islet-1, to mark the nuclei of the central RB neurons (Fig. 2A, 2C). At all three stages of larval development, there are more neurons in the most anterior region (somite 7). The numbers decrease as you move to more posterior regions. The spatial gradient persists throughout larval development. Particularly in the more anterior regions, the numbers of RB neurons decrease during larval development. This temporal pattern reflects a dynamic change in the population of RB neurons.

We also used Islet-1 immunocytochemistry to mark the nuclei of the peripheral DRG neurons at all three stages of development (Fig. 2 B-C). Unlike the RB neurons, the number of DRG neurons increases during this period of development. Only a couple of neurons are present in the most anterior region of some larvae at the earliest stage of development. There are still small numbers present one day later, but they are present at all axial levels. By the oldest stage, the numbers of cells are similar to those of RB neurons. At the oldest stage, there is a difference between the axial patterning of DRG and RB neurons. Although both have more neurons in the anterior versus posterior regions, there is a decrease or dip in the number of DRG neurons in the interlimb region (somite 10). The pattern of DRG sensory neurons differs from that of RB neurons, but both change in a dynamic way during the larval period of development.

We quantified sensory innervation at the three stages of development by measuring the total length of neurites innervating the somites (Fig. 2D). At the two earliest stages of development, there are roughly similar amounts of innervation in all three thoracic regions. Only the tail has reduced amounts of innervation at this time. By the oldest stage, the three posterior regions have undergone large increases in the amount

of innervation. This corresponds to an increase in the size of somites over the same period of time (not shown). The length of neurites forms a posterior to anterior gradient at this stage. The large increase in neurite length also illustrates the large amount of growth in sensory innervation of the skin that occurs during this period of development.

Denervation of the skin

The dynamic growth of sensory neurites could mean that they are relatively autonomous from their cell bodies. To test this, we denervated patches of skin for different periods of time to determine how long neurites persist in the skin. Before denervation (Fig 3A), neurites extend throughout the patch. However most of the neurites are gone only one hour after denervation (Fig 3B) to approximately 10% of the control level (Fig 3C). When neurites are separated from their cell bodies, they rapidly degenerate, indicating that during this period of rapid growth, they must remain intact and connected to their cell bodies.

Regeneration of skin sensory neurons

Following degeneration, we tested whether sensory neurites could regenerate into a denervated patch of skin. We transplanted a denervated patch of skin from a donor larva to a denervated site on a host. The skin was given time to heal and sufficient time (one day) for growth cones traveling at a normal rate (80 $\mu\text{m/hr}$; Roberts and Taylor, 1982), to easily traverse the patch. Examples of four different transplants are shown in figure 4. They include transplants of skin to animals of the same stage (isochronic, Figs. 4A and C) and transplants between animals at different stages (heterochronic, Figs. 4B

and D). Following isochronic transplantation, a greater area of the denervated skin was innervated than following heterochronic transplantation. Neurites entered the isochronic transplants from more places than heterochronic transplants. In all transplants, neurites could enter from any direction, but more seemed to enter from the dorsal side. Although the amount of growth varied, successful reinnervation occurred in all transplants.

We quantified the innervation of the transplants to determine if the two populations of neurons had a preference for innervating targets in the skin at the same stage of development. At 2.2 d, RB neurons are the only sensory neurons present in the host (Fig. 2) and they re-innervate 46.5 ± 8.9 % of the area in denervated skin from animals at the same stage of development (Fig. 5). The area of skin from older (7.5 d) animals re-innervated by younger RB neurons was significantly ($P < 0.05$) less than isochronic controls (21.4 ± 3.6 %; Fig. 5). At 7.5 d, sensory neurons have increased their innervation of the skin following DRG differentiation (Fig. 2). They re-innervate 49.0 ± 6.4 % of the area of skin from 7.5 d animals. However, those neurons re-innervate significantly ($P < 0.05$) less (24.6 ± 4.4 %) skin from younger (2.2 d) animals (Fig. 5). In 3.1 d hosts, there is an even greater area re-innervated in isochronic transplants (76.8 ± 8.6 %) than in either 2.2 d or 7.5 d isochronic transplants. This is similar to the area re-innervated in heterochronic transplants of older skin 75.1 ± 8.3 %. This is when DRG neurons are just starting to differentiate (Fig. 2B). Neurons from 7.5 d animals, which have RB and DRG neurons, do not re-innervate the large areas characteristic of neurons at 3.1 d, when RB neurons are younger and DRG neurons are just starting to differentiate. The results of all of these transplant experiments show that established RB or DRG neurons preferentially re-innervate targets at the same stage of development.

Pattern of sensory regeneration to the skin

During larval development, the pattern of innervation of the skin changes. Ciliated epidermal and Small Secretory (SSC) cells appear following neural tube closure, but begin to disappear as the animal approaches metamorphosis. Some cells that form a dispersed pattern like both of these epidermal populations, gradually become circumscribed by neurites (Fig. 1). However, this pattern changes as ciliated and presumably SSC cells disappear. To quantify this change, we measured the length of neurites in an area of skin from normal animals at different stages of development and counted the number of cells in the area totally surrounded by neurites. The number of encircled cells per unit neurite length in normal (control) animals is shown in figure 6. At 2.2 d, DRG neurons have not differentiated, so all of the skin sensory innervation is by RB neurons. The number of encircled cells per unit of neurite length is similar to that of animals at 3.1 d, which is when DRG neurons are just starting to differentiate. By 7.5 d, when RB neurons are older and some have started to die, the number of encircled cells per unit length significantly ($P < 0.05$) decreased. At 7.5 d, the number of ciliated epidermal cells also decreased (Nishikawa et al, 1992), as the larval skin replaces the pre-larval or embryonic skin. Presumably similar changes occur to the SSC cells at this time. Both sensory neurons and their targets change over this short period of developmental time.

The pattern of sensory innervation is maintained following regeneration into skin at the same stage of development. We hypothesized that skin transplanted to hosts at the same stage of development (isochronic transplants) would have the correct targets available to guide neurons and form a pattern similar to normal skin. Although the

number of encircled cells per unit neurite length in isochronic transplants at 2.2 d was somewhat lower than 2.2 d controls and 3.1 d isochronic transplants, both were still significantly ($P < 0.05$) greater than 7.5 d isochronic transplants (Fig. 6). Sensory neurites in both 3.1 d and 7.5 d transplants assumed patterns similar to controls. Although the pattern of innervation was not identical to controls, it was largely maintained after regeneration.

To determine whether the skin or the neurons direct the pattern of sensory innervation, we performed skin transplants between animals at different stages of development (heterochronic). What do RB neurons of a 2.2 d host do when they grow into skin from a 7.5 d donor? The pattern of encircled cells is similar to that of the host (2.2 d) and is significantly ($P < 0.05$) different from donor (7.5 d) skin (Fig. 6). This suggests that the RB neurons rather than targets in the skin are responsible for the encircling pattern. The reciprocal transplant of 2.2 d skin into a 7.5 d host should show the encircling pattern of 7.5 d sensory neurites, if neurons are responsible for the pattern. Although the average number of encircled cells was lower, the response was variable and was not significantly different from either the reciprocal transplant, the 2.2 d and 7.5 d controls or the 2.2 d and 7.5 d isochronic transplants. The wide variability means we cannot distinguish whether the neurons or their targets in this set of experiments stimulate the encircling behavior. We obtained similar results from heterochronic transplants between 3.1 d and 7.5 d animals. The encircled neuron pattern from 3.1 d host animals is similar to that of the 3.1 d control and isochronic transplants. It is significantly ($P < 0.05$) different from 7.5 d isochronic transplants, but not from 7.5 d controls. The RB and first DRG neurons rather than the targets in the skin, appear to be responsible for the

encircling behavior. However, transplants of 3.1 d skin onto an older (7.5 d) host, showed a lower level of encircling, but it was widely variable and was not significantly different from the reciprocal transplants, 3.1 d or 7.5 d controls or the 3.1 d and 7.5 d isochronic transplants. As with the heterochronic transplants between 2.2 d and 7.5 d animals, we cannot distinguish whether the neurons or their targets in this set of experiments stimulate the encircling behavior. Overall, the neurons in younger (2.2 d and 3.1 d) hosts encircle skin cells from an older animal in the same way that they would encircle skin cells from younger animals. The neurons from older animals have a more variable response to younger skin cells. This could be due to the fact that RB neurons remaining at this stage, regenerate differently from the large number of DRG neurons competing for targets in the skin.

Figure 1

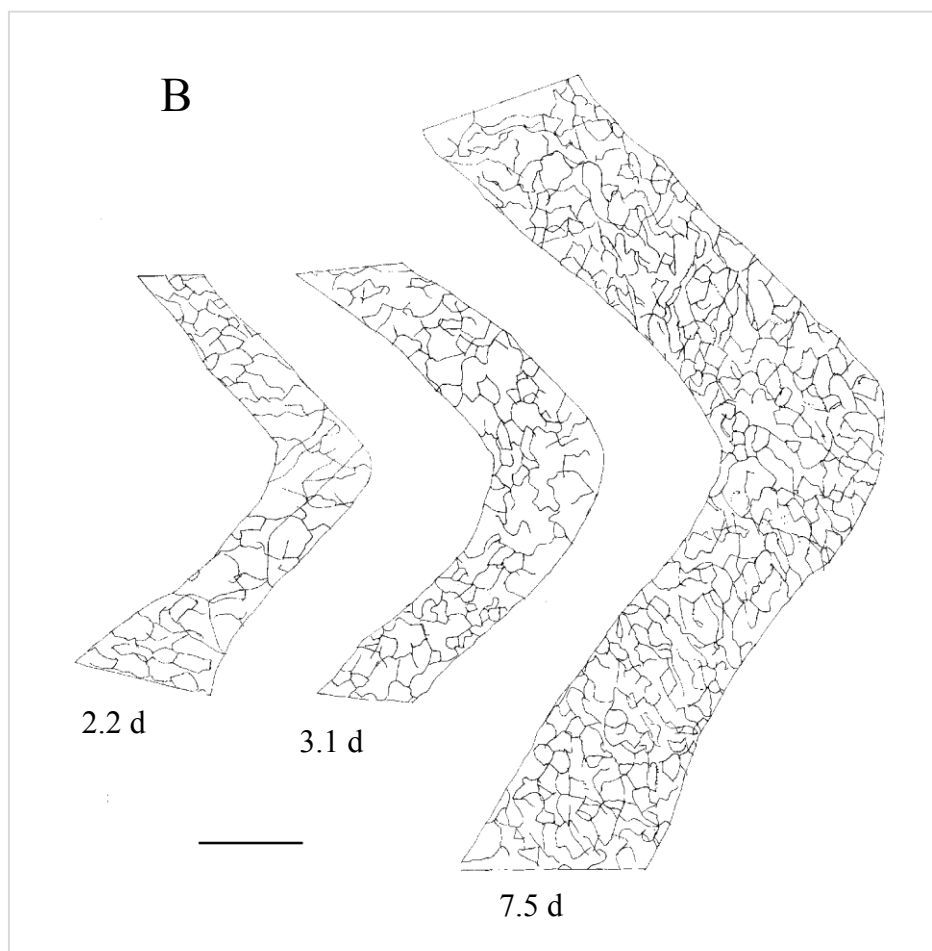
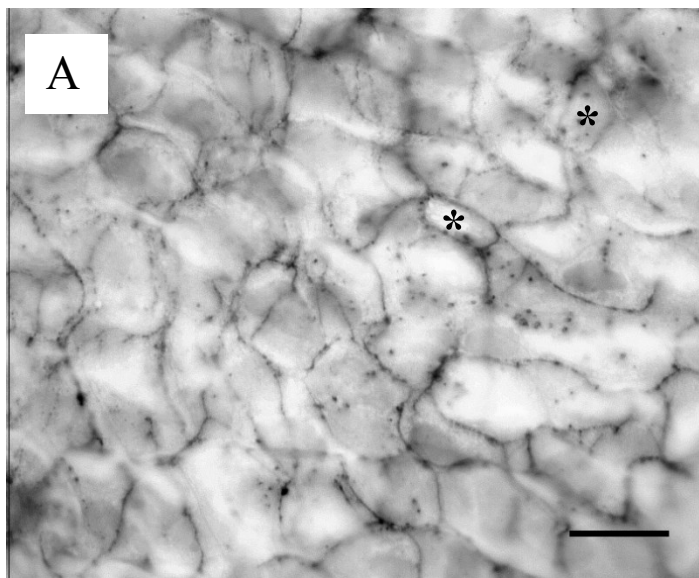


Figure 1: Development of sensory innervation in the skin. A. Photomicrograph of peripheral neurites expressing the HNK-1 epitope at 7.5 d, (st. 48). Some skin cells (black asterisks) are completely surrounded by sensory neurites. The neurites have multiple varicosities or boutons along their length. B. Camera lucida tracings of sensory neurites in the tail (somite 22) at hatching (2.2 d; st. 37/38), a mid-larval stage (3.1 d; st. 41) and the start of metamorphosis (7.5 d; st. 48) show that in the tail, there is dramatic growth during this period of development. Cal: A, 20 μm ; B, 25 μm .

Figure 2

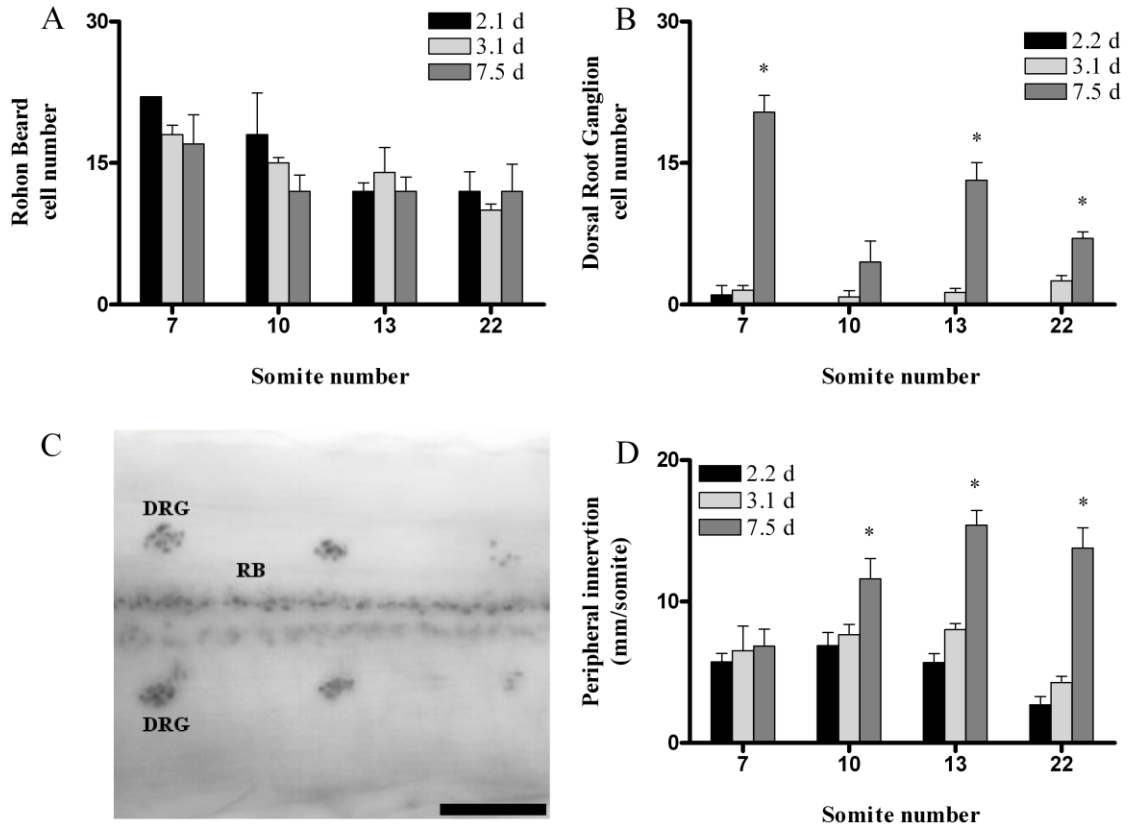


Figure 2: Differentiation of sensory neurons and their innervation of the skin. A. The number of Rohon Beard (RB) neurons associated with specific somites along the anterior-posterior axis, was quantified at three stages of development (hatching, mid-larval, start of metamorphosis). The somites are 7 (future fore-limb), 10 (inter-limb), 13 (future hind-limb) and 22 (tail). B. Only a few Dorsal Root Ganglion (DRG) neurons have differentiated in the most anterior region at 2.2 d (st. 37/38). New DRG neurons have differentiated by 3.1 d (st. 41) but there is no obvious pattern along the anterior posterior axis. Between 3.1 d (st. 41) and 7.5 d (st. 48) there is a significant ($P < 0.05$) increase in DRG number in all regions except the interlimb region (somite 10). C. Shows dorsal view of a 7.5 d (st. 48) spinal cord; both RB and DRG neurons are marked with Islet-1. D. Neurite length in the entire somite (peripheral innervation) is maintained between 2.2 d (st. 37/38) and 3.1 d (st. 41) in all three of the trunk regions, but is reduced in the tail. All but the future forelimb (somite 7) show a significant ($P < 0.05$) increase in neurite length between 3.1 d (st. 41) and 7.5 d (st. 48). Bars show mean and standard error of the mean (SEM). Statistical analysis utilized unpaired t-tests. Cal: A, 100 μm .

Figure 3

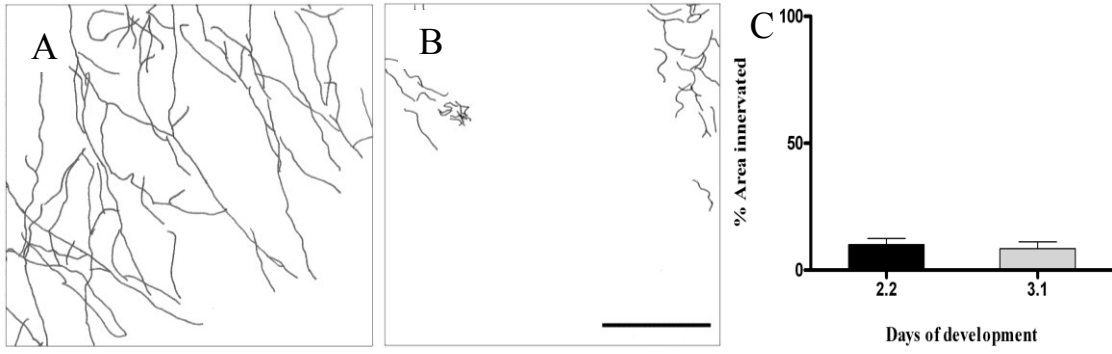


Figure 3: Rapid denervation of the skin. A patch of skin was removed from the trunk region of animals at 2.2 d (st. 37/38) and 3.1 d (st. 41) to denervate skin. A. shows a camera lucida tracing of the sensory neurites in skin marked with HNK-1 before denervation. B is a patch of skin removed one hr. after denervation from a 3.1 d animal. C. In both stages, almost 90% of the area in the patch became denervated within one hour. Bars show mean (n=3) and standard error of the mean (SEM). Cal: A, B, 100 μm .

Figure 4

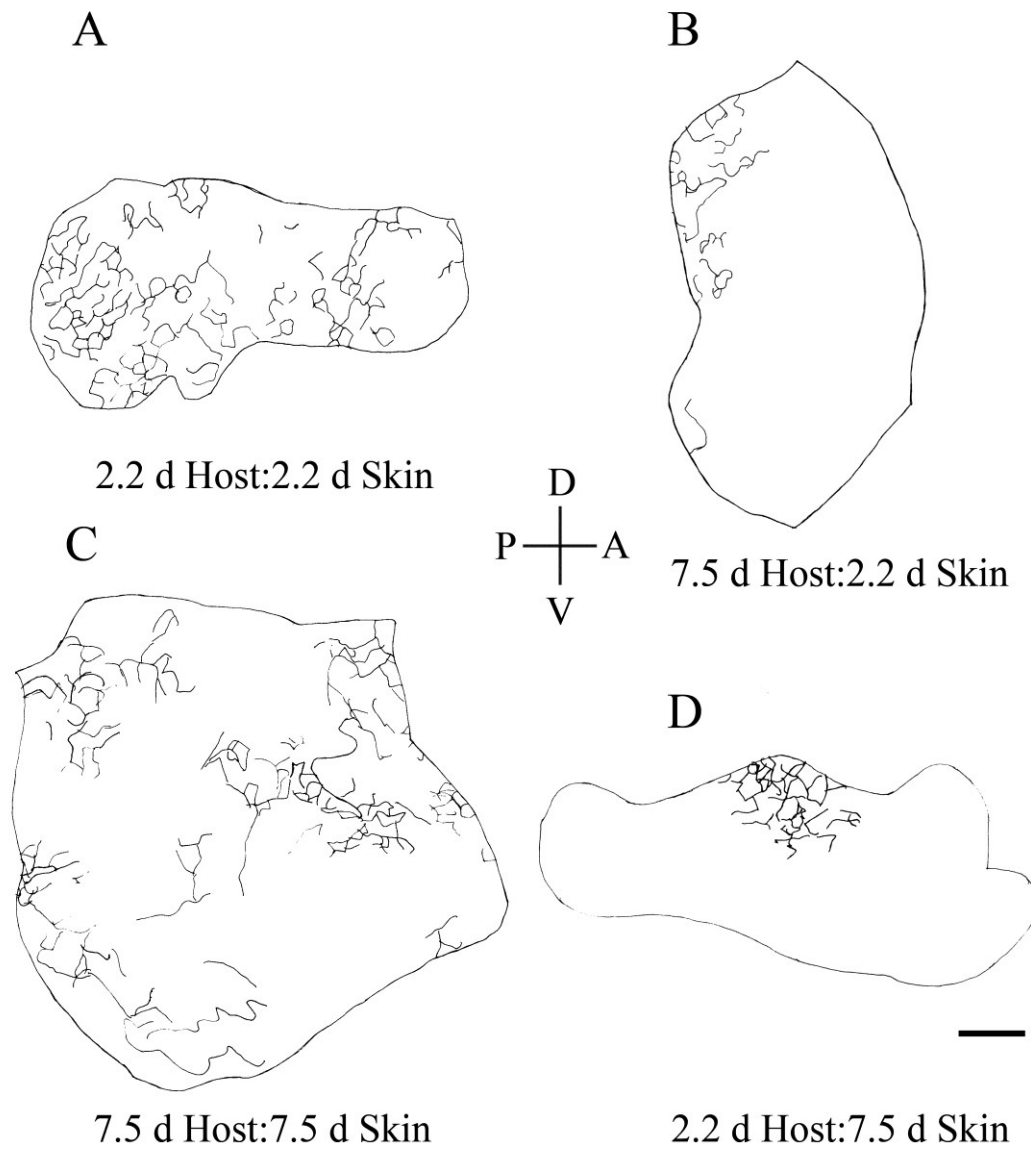


Figure 4: Regeneration of skin sensory neurites. Regeneration of neurites from 2.2 d (st. 37/38, post-hatching, A) and 7.5 d (st. 48, metamorphic, B) hosts into skin from a 2.2 d animal after regeneration for one day. Regeneration of neurites from 7.5 d (C) and 2.2 d (D) hosts into skin from a 7.5 d animal after regeneration for one day. Hosts regenerated greater lengths of neurites and innervated a greater percentage of the transplant area in isochronic transplants. Orientation of the transplants is indicated (D (dorsal), V (ventral), A (anterior), P (posterior)). Cal: 25 μ m.

Figure 5

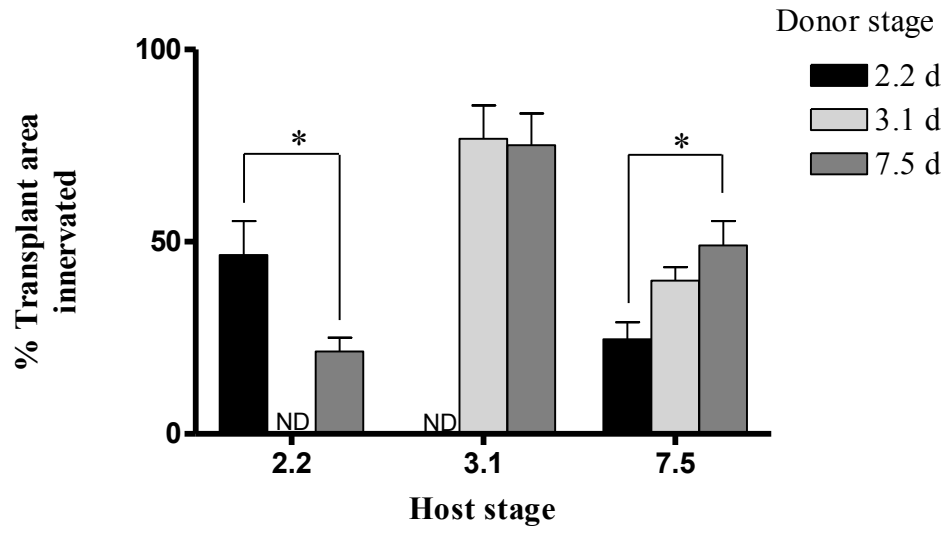


Figure 5: Area innervated by regenerating neurites. The area innervated by regenerating neurites was measured and expressed as the percent of the entire transplant area. For each stage, isochronic transplants showed the maximum amount of area covered by regeneration. For 2.2 d the percentage of the isochronic area innervated (black bar) was significantly ($P < 0.05$) greater than the heterochronic area innervated (dark gray bar). For 7.5 d hosts, the percentage of the isochronic area innervated (dark gray bar) was significantly ($P < 0.05$) greater than the heterochronic area innervated (black bar). Bars show mean and standard error of the mean (SEM). Statistical analysis utilized unpaired t-tests.

Figure 6

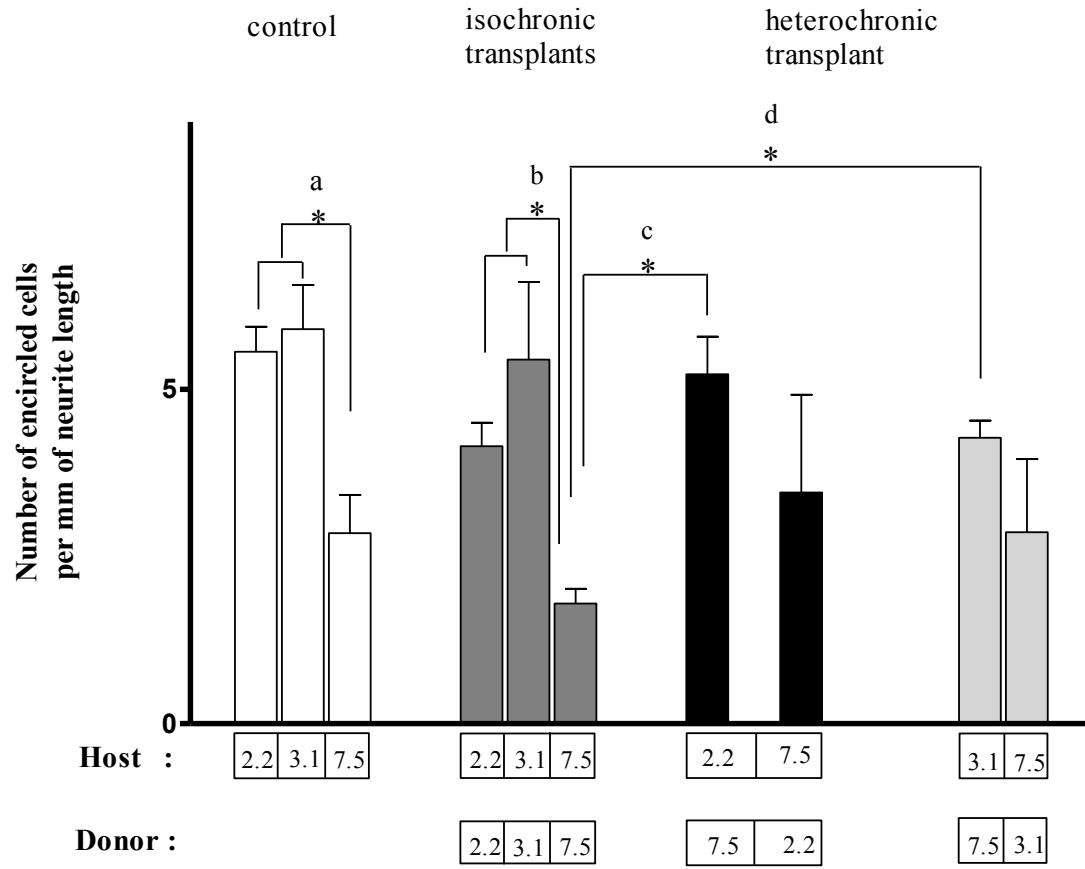


Figure 6: Pattern of regeneration. The total number of cells encircled per unit neurite length was quantified in control and transplant to test the pattern of regeneration. The total number of encircled cells in unoperated controls at 2.2 d and 3.1 d are both significantly greater (a; $P < 0.05$) than 7.5 d controls. The same holds true for isochronic transplants (b; $P < 0.05$). The number of encircled cells in heterochronic transplants from 7.5 d donor skin to 2.2 d hosts is similar to 2.2 d controls or isochronic transplants and different from 7.5 d controls or isochronic transplants (c; $P < 0.05$). The number of encircled cells in heterochronic transplants from 7.5 d donor skin to 3.1 d hosts is similar to 3.1 d controls or isochronic transplants and different from 7.5 d controls or isochronic transplants (d; $P < 0.05$). The results suggest that neurons from the hosts rather than targets in the donor skin are responsible for the encircling pattern. The total number of encircled cells in the reciprocal crosses between 2.2 d or 3.1 d donors to 7.5 d hosts are not significantly ($P > 0.05$) different from the 2.2 d/3.1 d/7.5 d unoperated controls, and 2.2 d/3.1 d/7.5 d isochronic transplants. Bars show mean and standard error of the mean (SEM). Statistical analysis utilized unpaired t-tests.

Table 1

Time	Stage	Behavior	Rohon Beard neurons	Dorsal Root Ganglion neurons	Skin changes
1.0 d	23				Differentiated ciliated cells appear ¹
1.2 d	26				Serotonin synthesis starts in the skin ²
1.8 d	33/34				Chromatophores differentiate ³
2.2 d	37/38	Post hatching	Max number present	None present	Ciliated cells stop differentiating ⁴
3.1 d	41	Mid-larval stage	Most present	First cells starting to differentiate	Ciliated cells begin to die ⁴ Lateral line starts to differentiate ³
3.3 d	42				Serotonin production stops ²
5.5 d	45-47				Most ciliated cells have disappeared ⁴
7.5 d	48	Start of metamorphosis	Have started to die	Large numbers present along the anterior/posterior axis	Lateral line is visible ³

1. Deblandre et al, 1999 2. Walentek et al, 2014 3. Nieuwkoop & Faber, 1994 4. Nishikawa et al, 1992.

Table 1: Sensory innervation by Rohon Beard and Dorsal Root Ganglion neurons at the three stages of larval development examined in this work. Changes in the skin are included to show how they correspond to changes in the sensory neurons.

Table 2
Donor (skin)

Host (neurons)	2.2d	3.1d	7.5d	
	2.2d	+ to +++	ND	+
	3.1d	ND	+++	+++
	7.5d	+	+ to ++	++ to +++

Table 2: Qualitative summary of neurite growth into skin transplants after one day of regeneration. + indicates some growth, while +++ indicates extensive growth. ND is not determined. In general, isochronic regeneration could be variable, but was better than growth into transplants from the most widely-separated ages. The number (n) for each combination is greater than or equal to 3.

Discussion

Sensory Neuron Development

In *Xenopus laevis* two distinct populations of sensory neurons mediate mechanosensory function during development. Rohon Beard (RB) neurons begin to differentiate during early gastrulation (Lamborghini, 1980) and send peripheral neurites to their epidermal targets (Roberts & Hayes, 1977; Somasekhar and Nordlander, 1997). RB neurons are present only in the embryonic and larval stages of development. They undergo programmed cell death and gradually disappear before the animal completes metamorphosis (Hughes 1957; Lamborghini 1987). The mechanosensory function of these neurons is eventually replaced by DRG neurons, which derive from the embryonic neural crest. DRG neurons begin to differentiate during early larval stages (Nieuwkoop & Faber, 1994) and thus overlap with the RB neurons. The mechanosensory DRG neurons innervate epidermis associated with Merkel cells. They also have many other targets, like proprioceptors, thermoreceptors and nociceptors. The larger array of sensory structures is used by both juveniles and adults to interact with their more complex environment.

We quantified the timing and patterning of sensory neurons during embryonic and larval stages. Table 1 shows the rationale for choosing the three specific stages of development. RB neurons appear in a anterior–posterior gradient during development. Our data shows that there are more neurons in anterior regions but they gradually decrease as you move to more posterior regions. In addition, the number of neurons at each axial level was maintained at all three stages of development (Fig 2A). As for DRG

neurons, very few are present at early larval stages but their number increases dramatically by the start of metamorphosis (Fig 2B).

Temporal and spatial changes of the two sensory neuron populations should be reflected in the innervation of their peripheral sensory targets. We expected to see an increase in peripheral innervation as the animal grew. Therefore we quantified neurite outgrowth during the transition from RB to DRG neurons. Our data shows that there is an increase in peripheral innervation as the animal grows. The increase in innervation is seen by the start of metamorphosis in posterior regions (Fig 2D). There is a large increase in the number of DRG neurons (Fig 2B), which could explain the increase in innervation. However the innervation by sensory neurons is not segmentally restricted and peripheral neurites branch extensively to innervate several segments along the length of the animal. Therefore the differential change in the number of DRG neurons at different axial levels is not correlated to the pattern of peripheral innervation. The increase in innervation of the tail is primarily due to the huge increase in target area as the tail somites rapidly expand in size. The absence of increase in innervation of the skin in the most anterior region at metamorphosis is because this somite reached its maximum size at the earliest stage and stopped growing.

The transition from RB to DRG and changes in peripheral innervation occur within a very short period during larval development when embryonic skin changes to larval skin and embryonic behavior becomes more complex after hatching. By transplanting skin between animals at different stages of development, we test the role of targets in directing outgrowth of the regenerating neurites present in the host. Our results also support that changes in the neurons occur during a switch in the mechanosensory

mechanisms during development and provide a candidate target for the outgrowing neurites.

Neurite Outgrowth

Development. RB and other central neurons begin extending neurites around the time of neural tube closure. The RB growth cones that extend into the periphery have been the subject of a number of *in vivo* studies (Roberts and Hayes, 1977; Roberts and Taylor, 1982; Roberts and Taylor, 1983; Taylor and Roberts, 1983). These studies used scanning electron microscopy to show the formation of the dense plexus of neurites under the developing skin. Their extensive characterization of growth cones, neurite behavior and the correlation to mechanosensory function, provides excellent background for our studies on growth at later stages and during regeneration of these neurons. Scanning electron microscopy provides ultrastructural detail, but is limited to neurites on the myotome surface and the inner surface of the basal lamina. Once the neurites penetrate the basal lamina to contact their epidermal cell targets, they cannot be visualized using this technique. In addition, before hatching, a collagen matrix starts to form, making it impossible to distinguish fine neurites from collagen fibrils (Taylor and Roberts, 1983) at later stages of development. Silver stains can show individual neurites (Hughes, 1957; Roberts and Hayes, 1977), but do not provide their density. Likewise, filling cells by applying crystals of Horse Radish Peroxidase (HRP) can show the complexity of individual neurite arbors, but not the density of many such arbors in the skin (Clarke et al., 1984). An immunocytochemical technique utilizing an antibody to the HNK-1 epitope present on neural cell adhesion molecules, allows the visualization of the sensory

plexus associated with the skin in embryonic animals (Somasekhar and Nordlander, 1997). We have modified this technique to mark sensory neurites at older stages. In our hands, the technique can quantitatively mark neurites associated with the epidermis, including endings between superficial cells and those on their basal surface. None of these processes can be visualized with scanning electron microscopy.

As RB neurons grow out of the neural tube, their substrate undergoes several changes. Initially, they grow over the myotomes, where growth cones are quite large, elaborate and have a ventral orientation (Roberts and Taylor, 1983). Then they move to the basal lamina underlying the epidermis and assume a more variable shape. Growth cones retain their ventral orientation and become simpler as they enter more ventral regions (Roberts and Taylor, 1983). In addition, the growth cone can follow other neurites and physical features of the substrate (Roberts and Taylor, 1983). Finally neurites enter holes in the basal lamina to contact epidermal cell targets (Taylor and Roberts, 1983). These holes are over the boundaries of epidermal cells, where they establish varicosities on the sides and basal surface of cells in the superficial layer (Fig. 1; Roberts and Hayes, 1977).

Regeneration. In our regeneration experiments, several parameters differ from those during development. First, the RB neurons are older and some have started to die. The potential to sprout growth cones and extend neurites could be quite different after their initial outgrowth. Second, the surfaces used by earlier growth cones have clearly changed. As the embryonic skin becomes larval skin, the composition of the basal lamina changes. For example, a fibrillar matrix composed of collagen forms on the basal lamina (Taylor and Roberts, 1983). Our experiments show that older neurites can sprout

and grow on these substrates. The reduced growth into isochronic explants may be due to discontinuity of the basal lamina in the host and donor tissue and presumably the secretion of new extracellular matrix molecules into the damaged region. In heterochronic transplants there would also be a reduced ability to grow across the boundary between host and donor, but the different composition of the basal lamina in the two tissues could further reduce growth into the transplant as seen in figure 4.

During development of the RB plexus, the initial pattern of growth can help explain the pattern of regeneration. RB growth cones extend over the anterior myotomes first, and then over the posterior myotomes and more ventral regions (Roberts and Taylor, 1982). Thus, there is an anterior/posterior gradient in the outgrowth of RB neurites. The growth cones move at a rate of approximately 80 μm per hour (Roberts and Taylor, 1982) or 2 mm per day. This means that any growth cones that could enter our explants should be able to easily grow throughout it. This coincides with our observations.

Studies of the orientation of RB growth cones in the myotomes and the skin basal lamina during initial outgrowth, can be similar to the neurite orientation during regeneration. The first wave of growth cones is oriented primarily in the ventral direction as they extend down from the dorsal neural tube (Roberts and Taylor, 1982). Analysis of the neurites, shows that they run from dorsal to ventral in the trunk and longitudinally (ie., anterior to posterior) in the tail because of the absence of RB neurons in the tail (Roberts and Hayes, 1977). Individual RB neurons innervate skin areas just posterior to the location of their cell body (Roberts and Hayes, 1977). In transplants, most neurites enter from the dorsal side and grow ventrally. Thus regenerating neurites

show a similar bias as the initial growth cones. They grow ventrally, even though there are plenty of neurites available to grow from all directions into the explant. Plenty of denervated targets are available in the explants, however nearby neurites infrequently deviate from their innate direction to innervate them. This basic orientation of regenerating sensory neurites was apparent in young animals, older animals and even in transplants between the two (Fig. 4). Thus, either the neurons or their substrate may have an inherent polarity that remains well after the first neurites have extended to their targets.

Regeneration of sensory neurites during larval life is remarkably rapid. Denervating epidermis by removing it from donor animals resulted in the loss of approximately 90% of sensory neurites within one hour (Fig 3C). Within 24 hours of transplanting them to a host animal, significant amounts of regenerated neurites were apparent. When compared to the regeneration of mechanosensory neurites in adult animals this is extremely fast. In adults, the mechanosensory neurons are in the DRGs. These neurons grow to Merkel cells in the skin (Scott et al., 1981). Following nerve section, local mechanosensitivity gradually decreases until it disappears after five to six days. Nonetheless, severed neurites can still conduct impulses if they receive a direct electrical stimulus (Mearow and Diamond, 1988). Following nerve section, mechanosensory responses were not present until three weeks after nerve section (Mearow and Diamond, 1988). Although we did not measure sensory responses, the initial disappearance of the neurites followed by rapid regeneration, indicates a dramatic difference between the two types of mechanosensory neurons and their associated structures in larval and adult animals.

Mechanosensory Function

Development. The mechanosensory system of *Xenopus* and other amphibians goes through several distinct changes during early development. The changes reflect the intimate relationship between the skin and sensory neurons. Following neural tube closure in *Xenopus*, the embryonic skin is capable of conducting impulses when it receives a noxious stimulus (Roberts, 1969). Recent experiments show that these impulses enter the central nervous system via the RB neurons for a brief period of developmental time (James and Soffe, 2011). At hatching, the animals have a dual mechanosensory system that responds to both noxious stimuli and light touch (Roberts and Smyth, 1974). As the mechanosensory endings of RB neurons differentiate, they no longer respond to skin impulses (James and Soffe, 2011), but to light touch from a small receptive field (Clarke et al, 1984).

Regeneration. As neurites regenerate into transplanted skin, they need to repair both mechanosensory systems. Regeneration of the noxious response would require integration of the transplanted patch of skin with that of the surrounding host skin, and the formation of the appropriate junctions to allow propagation of impulses across the patch. It also may transiently require RB neurons to directly transmit the nociceptive skin impulses before their mechanosensory endings differentiate and are capable of conducting light touch stimuli. This would be the same sequence that occurs during normal development. Regeneration of the light touch response (Clarke et al., 1984) would require the differentiation of new mechanosensory endings in the transplanted skin

We have not measured sensory responses, but we can make some conclusions based on the expression of neurite markers during regeneration. The reduced neurite

coverage of the transplanted tissue indicates a clear reduction in the ability to respond to touch during the day of regeneration following transplant. Furthermore, the reduction is not due to the age of transplanted skin, since isochronic transplants are innervated equally well at all three stages of development. Heterochronic transplants between 2.2 d (st. 37/38) and 7.5 d (st. 48) receive significantly less sensory neurite innervation than isochronic transplants (Table 2; Fig 5). Thus there appears to be two potential checkpoints limiting regeneration of the neurites. First, neurites do not have unrestricted access to the skin transplant at either the older or younger stage. This could be due to damage of the extracellular matrix during removal of skin from the donor and the host or from tissue changes at the site of annealing between donor and host tissues. Second, a neurite has an even greater difficulty in innervating denervated skin at a different age from itself. These stage-specific differences could be due to changes in the skin and the associated targets between the two stages of development.

Mature Mechanosensory System. The mechanosensory system changes as the animal matures. DRG nerve endings are associated with Merkel cells, which were originally described in amphibians (Merkel, 1880), but are conserved throughout the entire vertebrate lineage. The Merkel cell-neurite complexes respond to touch (Mearow and Diamond, 1988) in much the same way as RB neurons (Clarke et al, 1984). Likewise, the mechanism of transduction in both involves the excitation of free sensory nerve endings that penetrate the basal lamina underlying the skin (Roberts and Hayes, 1977; Mearow and Diamond, 1988).

The transition from the embryonic to the adult mechanosensory systems has not been quantified in *Xenopus*. Merkel cells are present in high density on the tentacles of

Xenopus tadpoles undergoing metamorphosis (Ovalle, 1979; Eglmeier, 1987). Tentacles become prominent by two weeks (st 50), but their rudiments are present before four days of development (st 44; Nieuwkoop and Faber, 1994). The development of Merkel cells in the larval and metamorphic skin has not been quantified in either *Xenopus* tentacles or skin. However, RB neurons are still present at st 50 (Lamborghini, 1987), which means that they co-exist with Merkel cells.

Merkel cells derive from the epidermis, since they can differentiate under aneurogenic conditions (Tweedle, 1978; Mearow and Diamond, 1988). Likewise the cells innervated by RB neurons are all derived from the epidermis (Drysdale and Elinson, 1992; DeBlandre et al, 1997). Sensory neurites form complexes with synaptic-like endings on Merkel cells, but Merkel cells in *Xenopus* are not needed to produce a normal mechanosensory response (Mearow and Diamond, 1988). Rather, the evidence supports a role for Merkel cells as a target for mechanosensory nerves in amphibians (Scott et al., 1981). In this way, the Merkel cells determine the distribution of mechanosensory nerves in the skin. Likewise, specialized epidermal cells in the embryonic and larval skin, are initially the targets of mechanosensory RB neurons (Somasekhar and Nordlander, 1997). All of the nerve endings are in the skin (Roberts and Hayes, 1977; Taylor and Roberts, 1983) and the pattern of encircled cells (Somasekhar and Nordlander, 1997) is dispersed, like that of ciliated epidermal cells (see DeBlandre et al., 1999) and conical or small secretory cells (SSC) cells. The underlying molecular mechanism patterning ciliated epidermal cells and presumably SSCs, involves Notch/Delta signaling together with a subsequent step regulating intercalation of the cells moving from the germinal layer into the outer epidermal layer, to effectively disperse them across the skin (DeBlandre et al,

1999). Little is known about the mechanism patterning Merkel cells, although in *Xenopus*, 2-4 of them differentiate around the opening of cutaneous skin gland ducts (Mearow and Diamond, 1988). This also provides a dispersed pattern across the skin, but since the cells occur in groups, rather than as individual cells, like ciliated epidermal and SSC cells, their dispersion likely has a different mechanism.

Development of Skin Sensory Targets

Recent work provides a hint of the nature of the embryonic targets of RB neurons. Somasekhar and Nordlander (1997) described a class of epidermal cells that did not have cilia that they named “conical cells” based on their shape. The cells assumed a dispersed distribution in the skin that was similar to the ciliated epidermal cells (see Deblandre et al., 1999). Experiments have identified a new cell type in the skin. Like the conical cells, it does not have cilia and forms a dispersed pattern, suggesting that these two cells are the same. The recent description also shows that the cells develop large granules that are secreted and therefore call them Small Secretory Cells (SSCs; Dubaissi et al., 2014; Walentek et al., 2014). The granules contain serotonin and a mucous-like substance that has been named “otogelin-like”.

There is an extensive body of literature on the developmental actions of serotonin. Among them, it can direct the migration of neurons in *C. elegans* (Kindt et al., 2002), and striatal and cortical axons in mammals (Speranza et al., 2013). In *Xenopus* skin, genes involved in serotonin production begin to be produced by all epidermal cells soon after neural tube closure (st 23; Walentek et al., 2014) and right after the initial appearance of RB growth cones. However, the large granules characteristic of SSCs, do not become

evident until st 32. Enzymes responsible for serotonin synthesis stop being produced at st 42 (Walentek et al., 2014), shortly before RB neurons start their prolonged period of cell death (Lamborghini, 1987). Thus, low levels of serotonin produced by the epidermis following neural tube closure, could stimulate the initial phase of RB neurite outgrowth. As the conical or SSC cells form, growth cones could be attracted to serotonin released by these cells that form a dispersed pattern in the epidermis. The serotonin could also stimulate penetration of the basal lamina and differentiation of sensory nerve endings around the cells. This would explain why the cells that are entirely encircled are not as numerous as the other dispersed population of cells in the superficial epithelium: ciliated epidermal cells.

The hypothesis of a chemoattractive role for serotonin in the skin and the SSCs suggests an explanation of how the embryo can ensure even mechanosensory innervation across its body. The original hypothesis explaining the encircling behavior by Somasekhar and Nordlander (1997) was that neurites were attracted to the conical/SSC cells by the absence of chondroitin 6-sulfate on their surface, while it was present on the surface of other epidermal cells. However, they also needed to propose that the growth inhibitory effects of chondroitin 6-sulfate were transient, since its expression does not change over the time the rest of the epidermal cells become innervated.

A chemoattractive role of a skin cell for mechanosensory innervation is consistent with the role of Merkel cells in attracting DRG neurites in adults (Scott et al., 1981). The conical/SSC cells could attract RB neurites and promote the differentiation of mechanosensory specializations. Lower concentrations of serotonin in cells distal to the SSCs, would have fewer mechanosensory specializations and would not be encircled by

neurites. This model lends itself to a relatively simple test. Embryos could be incubated in a serotonin antagonist to test whether it would reduce a.) neurite outgrowth, b.) encirclement of cells in the epidermis and c.) the formation of specialized mechanosensory endings. It would also be necessary to show that cells containing serotonin are those that are encircled by RB neurons. Together these experiments could provide strong supporting evidence for a chemoattractive role of serotonin in the formation of the mechanosensory system of *Xenopus*.

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